In Vitro Techniques for the Assessment of Neurotoxicity

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Risk assessment is a process often divided into the following steps: *a*) hazard identification, *b*) dose–response assessment, *c*) exposure assessment, and *d*) risk characterization. Regulatory toxicity studies usually are aimed at providing data for the first two steps. Human case reports, environmental research, and *in vitro* studies may also be used to identify or to further characterize a toxic hazard. In this report the strengths and limitations of *in vitro* techniques are discussed in light of their usefulness to identify neurotoxic hazards, as well as for the subsequent dose–response assessment. Because of the complexity of the nervous system, multiple functions of individual cells, and our limited knowledge of biochemical processes involved in neurotoxicity, it is not known how well any *in vitro* system would recapitulate the *in vivo* system. Thus, it would be difficult to design an *in vitro* test battery to replace *in vivo* test systems. *In vitro* systems are well suited to the study of biological processes in a more isolated context and have been most successfully used to elucidate mechanisms of toxicity, identify target cells of neurotoxicity, and delineate the development and intricate cellular changes induced by neurotoxicants. Both biochemical and morphological end points can be used, but many of the end points used can be altered by pharmacological actions as well as toxicity. Therefore, for many of these end points it is difficult or impossible to set a criterion that allows one to differentiate between a pharmacological and a neurotoxic effect. For the process of risk assessment such a discrimination is central. Therefore, end points used to determine potential neurotoxicity of a compound have to be carefully selected and evaluated with respect to their potential to discriminate between an adverse neurotoxic effect and a pharmacologic effect. It is obvious that for *in vitro* neurotoxicity studies the primary end points that can be used are those affected through specific mechanisms of neurotoxicity. For example, *in vitro* systems may be useful for certain structurally defined compounds and mechanisms of toxicity, such as organophosphorus compounds and delayed neuropathy, for which target cells and the biochemical processes involved in the neurotoxicity are well known. For other compounds and the different types of neurotoxicity, a mechanism of toxicity needs to be identified first. Once identified, by either *in vivo* or *in vitro* methods, a system can be developed to detect and to evaluate predictive ability for the type of *in vivo* neurotoxicity produced. Therefore, *in vitro* tests have their greatest potential in providing information on basic mechanistic processes in order to refine specific experimental questions to be addressed in the whole animal. — *Environ Health Perspect* 106(Suppl 1):131–158 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-1/131-158harry/abstract.html*

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Introduction

Principles of Neurotoxicity Risk Assessment

Neurotoxicology is the study of the adverse effects of chemical, biological, and certain physical agents on the nervous system and/or behavior during development and in maturity. The topic of risk assessment in noncancer end points including neurotoxicology has received much attention and has led to the development of a systematic scientific and administrative framework to assess risk associated with exposure to chemical and physical agents (*1*). Risk assessment is defined as "the characterization of the potential adverse health effects of human exposures to environmental hazards" (*2*). The process of risk assessment is often divided into the following steps: *a*) hazard identification, *b*) dose–response assessment, *c*) exposure assessment, and *d*) risk characterization. The initial step in the risk assessment process is the identification of hazard. Hazard is defined as the likelihood that injury will occur in a given situation or setting. Human case reports, experimental laboratory animal studies, environmental research, and *in vitro* studies can all be used to identify toxic hazards. These studies can also be used to characterize the dose–response relationship between chemical exposure and toxicity. This information is critical in the identification of risk, which defines the likelihood of an adverse effect occurring under actual exposure conditions. Another important step in the risk assessment process is the determination of a quantitative estimate of risk. These estimates are derived from the extrapolation of experimental dose–response models obtained from animal or human studies to predict the type and estimate the extent of health effects in humans resulting from anticipated human exposure. Throughout

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Abbreviations used: ACh, acetylcholine; 5-HT, serotonin; AChE, acetylcholinesterase; BBB, blood–brain barrier; CNS, central nervous system; DMSO, dimethyl sulfoxide; E12, embryonic day 12; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GGTP, γ-glutamyl transpeptidase; LDH, lactate dehydrogenase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, *N*-methyl-4-phenylpyridinium ion; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; NRC, National Research Council; NTE, neuropathy target esterase; OPIDN, organophosphate-induced delayed neuropathy; Pb, lead; PC-12, pheochromocytoma cell line; PCB, polychlorinated biphenyl; PNS, peripheral nervous system; RNA, ribonucleic acid; TH, tyrosine hydroxylase; TMT, trimethyltin; VEC-DIC, video-enhanced contrast differential interference contrast microscopy.

these steps, research needs are identified from the risk assessment process (*3*).

Principles of the Nervous System—Potential Sites of Neurotoxic Attack

The nervous system comprises two components: the central nervous system (CNS), which is composed of the brain and the spinal cord, and the peripheral nervous system (PNS), which comprises the ganglia and the peripheral nerves lying outside of the brain and spinal cord inclusive of the autonomic nervous system. Two general types of cells are predominant in the nervous system: neurons and neuroglial cells (oligodendrocytes, astrocytes, microglia; and in the PNS, the Schwann cells). Neurons are similar to all other cells of the body in general structure, but they have additional anatomical features of axons and dendrites that allow conduction of nerve impulses for communication with other neural cells and between neuronal populations. The dendrites are elongated processes that emanate from the cell body and increase the neuronal surface area available to receive input from other sources. The surface area of the axonal process can be tens to thousands times greater than the cell body diameter and is specialized for the conduction of nerve impulses away from the cell body toward the terminal synapse and other cells (neurons, muscle cells, or glandular cells). The neuronal cell body synthesizes the necessary components for maintaining structural components of the membrane (e.g., proteins and lipids) and general cell functioning. Many axons are surrounded by layers of membrane from the cell processes of glia cells (the myelin sheath). Oligodendrocytes in the CNS and Schwann cells in the PNS form this myelin sheath. The myelin extension from any one cell covers only a short length of the axon, requiring a continuous series of glial cells to ensheath an entire axon. The presence of the myelin lamella allows for the acceleration of the nerve impulse by permitting salutatory conduction between the myelin sheath of each glia cell identified as the node of Ranvier. At each node the axon is nonmyelinated yet enveloped by teloglial cells. Although nonmyelinated axons are devoid of myelin lamella, they are ensheathed by plasmolemma of the similar cells.

Neurons are highly specialized cells and are responsible for the reception, integration, transmission, and storage of information. Afferent neuronal pathways carry information into the nervous system for processing; efferent pathways carry commands to the periphery. In addition, there are interneurons that process local information and transfer data within the nervous system. Within the CNS, neurons are segregated into functionally related nuclei that form interconnecting bundles of axonal fibers. Higher organizational levels consisting of several functionally related neurons are frequently called systems, e.g., motor, visual, associative, and neuroendocrine systems. The CNS consists of a number of systems responsible for the coordination of receiving and processing information from the environment, maintaining the balance of all other organ systems, and responding to changes in the environment.

The *in vivo* reactions of neurons to injury vary dramatically. Degeneration can be induced by a direct action on the perikaryon or loss of synaptic target site influences and deprivation of trophic factors. A number of chemicals appear to have distinct cellular specificity for neuronal populations. Although specificity can exist and the pattern of degeneration has been used in diagnostic neuropathology, degeneration of any particular neuronal type cannot necessarily identify the damaging agent. Often this pattern reflects the severity and duration of the injury and the acuteness or chronic nature of exposure. The degenerative process of the nerve cell can be either relatively quick or a slow, prolonged process, depending on the underlying mechanism.

Unlike neurons, glial cells have no true synaptic contacts; however, receptors for several neurotransmitters are present and functional on various glial cells. Cell–cell contacts exist between the glial cells and neurons that may regulate both neuronal and glial differentiation and are critical for the glial-guided migration of neurons during development. Glial cells have a dynamic nature and provide critical processes necessary to maintain normal functioning of the nervous system (e.g., regulation of local pH and ionic balances, and tropic support for neurite extension in the form of growth factors and cell adhesion factors). They can also be the target for a toxic response. For either the oligodendrocyte or the Schwann cell, the myelin membrane is vulnerable to numerous substances, toxic agents, and demyelinating autoimmune diseases such as multiple sclerosis and Guillian-Barre syndrome that specifically target and break down the myelin sheath. After injury to the nervous system, the astrocyte and the microglia both display profound responses. The astrocyte responds by proliferation or a morphological change characterized by cellular hypertrophy. This process of reactive gliosis has been proposed as an early marker of damage to the nervous system and can be detected morphologically or quantified by measurements of an astrocyte-specific protein, glial fibrillary acidic protein (GFAP). The sequence of the response can be rapid and transient or progressive and sustained over an extended period of time. The microglial cell assumes an immunologic role and is seen in the normal brain in a resting state; however, with injury it can become mobile and assume macrophage-like characteristics. In the PNS, this phagocytic role is provided by the resident macrophage as well as by the infiltrating peripheral macrophages. In a toxicant-induced response of the nervous system, each cell type may contribute to the overall manifestation of neurotoxicity not only individually but based upon complex cell–cell interactions altered or initiated during the injury response.

Role of *in Vitro* Systems in Determining Neurotoxicity

In the field of neurobiology, *in vitro* cell culture techniques have been successfully developed and employed to address specific questions of cell biology and nervous system functioning and provide a means to systematically study complex nervous systems (*4–8*). Such systems have two major and critical functions. First, they teach neuroscientists and neurotoxicologists the complexity of cellular functions of CNS elements. Second, they provide a convenient experimental tool for testing possible functions or postulates *in vivo* that otherwise might not be conducted (*9,10*). Cells harvested directly from the organism, dissociated into single cells before seeding into the culture vessel, and maintained *in vitro* for periods exceeding 24 hr are defined as primary cell cultures. If tissue fragments are maintained *in vitro*, the culture is referred to as an explant culture. Cell lines are cultures that have been serially transplanted or subcultured through a number of generations and can be propagated for an extended period of time. In neurobiology, *in vitro* methods are not ordinarily considered as alternatives to *in vivo* procedures. Instead, *in vitro* methods are selected to address specific hypotheses. Studies with tissues, cells, or cell fragments may provide the most appropriate approach and in many cases cannot be conducted in live animals. Therefore, *in vitro* models are used in an attempt to study biological processes in a more isolated context or in

the direct investigation of specific biological processes. It is generally recognized that *in vitro* systems often provide only partial answers to more complex problems; therefore, they can supplement, but rarely replace, investigations with whole animals. For many questions in biology and medicine, *in vitro* techniques may not be suitable. For example, *in vitro* techniques offer little information about a chemical effect on sensory or cognitive function. If one is interested in investigating the effects on the central processing of a message received by receptor cells, storage of this information in short- and long-term memory, or behavioral and somatic responses resulting from some of the sensory perception, assessment of the whole animal is essential.

Various types of *in vitro* approaches produce data for evaluating potential and known neurotoxic substances, including primary cell cultures, cell lines, and cloned cells. Although such procedures are important in studying the mechanism of action of toxic agents, their use in hazard identification in human health risk assessment has not been widely accepted. For example, the proposed guidelines for neurotoxicity risk assessment published by the the U.S. Environmental Protection Agency (U.S. EPA) (*11*) raise concerns about the ability of *in vitro* techniques to predict the neurotoxicity of various agents found in humans and animal models. This validation step requires considerations in study design, including defined end points of toxicity and an understanding of how a test chemical would be handled *in vitro* as compared to the intact organism. The neurotoxicity risk assessment guidelines propose that demonstrated neurotoxicity *in vitro* in the absence of *in vivo* data is suggestive, but inadequate evidence of a neurotoxic effect. Thus, the *in vitro* data is used often to enhance the reliability of *in vivo* data.

Use of *in Vitro* Techniques for Evaluating Neurotoxicity

Rationale

The rationale for the use of *in vitro* procedures to assess neurotoxicants is based upon a clear understanding of mechanistic processes underlying normal nervous system functioning and some forms of dysfunction. When applied to chemical-induced perturbations of the nervous system, knowledge of biological mechanisms has led to the examination of more sophisticated and subtle biologically based expressions of neurotoxicity. This advance has been seen in all levels of examination, from the integrative functioning of the nervous system as manifest by behavioral alterations, to morphological and biochemical alterations in the intact animal, to the molecular mechanisms associated with injury response or development, and to the intricate cellular changes examined by *in vitro* techniques.

Although the assessment of neurotoxic end points in the whole animal are presumed to be causally related to those initiated at the cellular level, in many cases the cascade of effects is not well understood. Chemicals rarely if ever affect all neurons indiscriminately but instead induce selective damage. Cellular toxicity depends on the specific sensitivity of the cell as well as on the extracellular concentration of the test compound. The effects seen may not be due to the primary toxicant administered but rather the result of bioactivation with subsequent response to a metabolite. In addition, toxic responses of neurons *in vivo* may be the result of toxicity of nonneuronal or nonneural cells, e.g., glial cells, endothelial cells. Observed responses may even be due to a systemic biological process activated by exposure such as hepatic encephalopathy or may be observed only with maturation or aging of the system. These features create a major problem in selecting an *in vitro* system that will model the nervous system target site or process for any particular chemical. With the use of *in vitro* systems, it must be kept in mind that no existing *in vitro* system will be able to measure all chemically induced effects. This limitation is due partly to the lack of all possible target sites and the synergistic interaction between the cell types. In addition, the limited culture life may not allow for the appearance of delayed responses or allow for discrimination between transient and persistent effects.

Many chemicals are introduced for industrial use with limited knowledge of how they might affect a biological organism. Alteration in structure of industrial or agriculture chemicals may appear trivial from a chemical perspective, e.g., addition or substitution of a methyl group, but that same change may have dramatic consequences with regard to activity in biological systems. In general, safety testing is designed to reduce unnecessary exposure of human and other animal populations. Thus, the optimal system for hazard identification would be the one most closely resembling the phenotype of concern in biochemical, physiological, molecular, and other cellular and organ processes. Dose-response data obtained from *in vitro* studies must be used with caution because of significant differences in the pharmacokinetic behavior of chemicals observed *in vivo* and *in vitro*. In addition, effects observed *in vitro* are not necessarily predictive of whether an adverse effect will occur following exposure *in vivo*. However, mechanism(s) of neurotoxicity identified using *in vitro* studies may contribute to determining whether an effect observed *in vitro* may also result in an adverse effect in animals or humans following chemical exposure.

Advantages and Disadvantages

The use of *in vitro* systems for toxicity testing has been discussed in numerous review articles (*12–18*). In each of these reviews, a number of various advantages and disadvantages have been identified (Table 1).

Access to the Cellular Environment. The physicochemical environment of cells is easily manipulated *in vitro*. Substances can be added or withdrawn from the culture medium, allowing precise temporal analysis of the sequence of events. The concentration of the test chemical can be controlled in terms of the amount being delivered to the entire cell population or to an individual target cell; however, this concentration must be consistent with the *in vivo* level of exposure to be meaningful. In

Table 1. General advantages and limitations of *in vitro* studies in neurotoxicology.

Access to the cell environment
Advantages
Ability to expose cells directly to a chemical
Ability to manipulate environmental conditions to augment, block, or modulate cell response
Evaluation of intrinsic cell response of cell to chemical
Ability to measure responses in absence of
Chemical metabolites
In vivo homeostatic mechanisms
In vivo filtering processes
Limitations
Chemical properties influencing bioavailability
Solubility
Volatility
Precipitation
Direct chemical interactions altering culture condition
pH
Osmolarity
Protein binding
Lack of *in vivo* chemical disposition factors
Lack of *in vivo* chemical metabolism
Lack of *in vivo* filtering mechanisms
Blood–brain and blood–nerve barriers

addition, the compound should be presented to the cells in a physiological formulation. Techniques exist that allow the direct injection of a substance into a cell and examination of the resulting cellular responses. However, the use of such techniques is limited to specific experimental questions, given the nonphysiological administration, the possible localized distribution of the chemical, and the injury induced by the injection alone.

Physicochemical properties of chemicals, such as solubility, volatility, pH, binding to components of the culture medium including protein binding, and osmolarity, must be considered prior to use in culture systems. It is difficult to evaluate *in vitro* chemicals that are either insoluble in aqueous systems or at neutral pH, thus forming insoluble particulate or precipitate over time. To expose cells to such insoluble compounds, additives like dimethyl sulfoxide (DMSO), Tween-80, or ethanol, are often added to the culture medium and could distort the toxicological characteristics. Some investigators have attempted to overcome these problems by solublizing the compounds using physiological carriers such as albumin, lipoproteins, or a specific carrier. Many other chemicals may be volatile and evaporate quickly. Such physiochemical properties present major problems in terms of obtaining adequate and constant concentrations of the compound of interest. The chemical nature of the substance administered must be considered. Alterations in the osmolarity or pH shifts in the culture medium can occur that are toxic to the cells or could stimulate the precipitation of nutrients from the culture media. The addition of a test chemical to the nutrient fluid could result in a direct reaction between the test compound and a component of the culture medium (e.g., protein denaturation, precipitation), possibly affecting the availability of the toxicant or essential nutrients, and modifying signals to the cultured cells. Any protein-binding properties of test chemicals could directly alter the microenvironment of cultured cells in a fashion that may not occur *in vivo*.

Chemical Disposition and Metabolism. Toxicokinetics consists of absorption, distribution, metabolism, and excretion of chemicals and their metabolites following exposure. The dose needed to induce toxic effects depends on these pharmacokinetic parameters. For example, the route by which a chemical enters the body can substantially alter the quantity absorbed and consequently modify the dose required to cause toxicity. Metabolism at the site of absorption (e.g., gastrointestinal tract, skin, lung) may determine the quantity of the compound reaching the circulatory system and the type and concentration of possible toxic metabolites. The absorbed compound may be distributed throughout the body by the circulatory system. Within the organism, the compound may be bioactivated and/or detoxified before it is released back into the circulation. Such metabolism and biotransformation of foreign compounds in the body can be affected by various enzymatic processes. The specificity and velocity of each are strongly species dependent (*19*). The rate of removal of a compound depends not only on metabolism but also on the excretion rate; large species differences in steady-state levels can occur due to differences in elimination rate. Differences in drug metabolism and excretion are widely considered to be an important reason for the large species differences observed in both dose and type of toxic response following drug or chemical exposure.

In culture, a test compound either remains unaltered or is relatively slowly modified, and one can examine the intrinsic toxicity of a substance to a cell in the absence of any metabolites. Many compounds require metabolic activation; others are detoxified by metabolism. For example, methyl *n*-butyl ketone is metabolized to the active compounds 2,5-hexanedione and 2,5-hexanediol. Xenobiotic inactivation systems are expressed in neural tissue, predominantly in nonneuronal cells (*20*). In addition, blood enzymes such as organophosphate hydrolyzing plasma esterase can be introduced via serum supplementation of growth media. The combined effect of these modifications can lead to unanticipated reductions in the actual concentration of the test agent. It is critical to understand the *in vivo* metabolism, distribution, and effects of potential metabolites in order to interpret data obtained from both *in vivo* studies and tissue culture systems. If the neurotoxic potential of a chemical *in vivo* is dependent upon metabolic parameters such as biotransformation, lipid–aqueous partitioning, and distribution, then the expression of toxicity in a culture system may be meaningless when related to whole-animal studies.

METABOLIC ACTIVATION SYSTEM. For certain classes of compounds (e.g., cyclophosphamide activated by cytochrome P450-dependent monooxygenase enzymes), the inclusion in the cell cultures of a metabolic-activating system is required to obtain toxic responses. Any artificial system used to simulate normal metabolism of chemicals should preferably be comparable to the *in vivo* condition. Usually, biochemical activation and detoxification of test substances are simulated *in vitro* by the addition of enzyme preparations, microsomal additives (e.g., embryo extract or S9 liver fractions), or metabolically active cells; however, the biotransformation processes may be incomplete or proceed by pathways different from those operating in intact organisms. Inclusion of a metabolic activation system, whether a liver microsomal fraction, embryonic microsomes, or isolated cells, requires a consistent source of extract to ensure reproducibility of experimental data. The metabolic pattern is determined by the type of metabolic system and the species and sex from which the metabolic competent cells are isolated. However, it can never take into account the polymorphisms seen for a variety of enzymes, some of which may be relevant to neurotoxicity (e.g., cytochrome P450s or monoamine oxidase [MAO-B]). It also requires the use of a number of animals to generate a bulk preparation for continued use. Results from studies using metabolic activation system must be interpreted in light of known cytotoxicity resulting from the addition of hepatic S9 fractions to neural cultures (*21*). In many cases metabolism is not limited to the liver, but also can include metabolism by resident cells in the organ tissue of interest. For example, in the nervous system, there is evidence that glial cells are necessary to produce the neurotoxic metabolite *N*-methyl-4-phenylpyridinium ion (MPP^+) from the parent compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (*22*), to convert glutamate to glutamine (*23*), and to sequester heavy metals from the neural environment (*24*). Thus, *in vitro* models in which the influences of extraneous chemical substances are studied are often deficient in competent metabolic systems operating in the whole organism. Although cell culture allows a precise determination of the toxic exposure delivered to cells, it also poses a difficulty in defining a toxicologically relevant dose.

EXPOSURE DURATION. Chemicals can have various toxic effects on the whole organism, depending on the exposure dose and duration. Toxic effects may be seen shortly after the initiation of exposure (acute effects) or only after some delay. In some cases, signs of neurotoxicity may be observed only after repeated or prolonged

exposure. The appearance of such effects may not only depend on the type of chemical and dose used, but may also depend upon the biological processes underlying the toxic response. Certain compounds interact directly with accessible cell components such as the cell membrane or vital enzymes. As a result, these structures will be functionally affected shortly after exposure. For these effects, the peak concentration and exposure schedule are of utmost importance. A delay in the appearance of effects is due to the initiation of an irreversible cascade of reactions, after which cell damage is manifested. Each step within the cascade, however, needs time to initiate the subsequent step, resulting in a delay in the appearance of the toxic response. Progressive effects are induced by the initiation of a cascade of reactions similar to delayed effects; however, the first changes can be observed only after a significant latency from which the damage is progressive intensified. In other cases, a toxic effect may require an accumulation of a chemical within the system for an extended period of time rather than a peak serum concentration. An example is bismuth, which shows no acute effects either *in vivo* or *in vitro* even at extreme doses/concentrations. Adverse effects become manifest only after a prolonged exposure period (*25*).

Depending on the type of *in vitro* system, neural cells may survive only a few hours or several months; however, each cell type can mature during this period. As a consequence, the exposure window differs among the different culture systems because of differences in survival. The majority of culture systems employed are applicable for examining acute responses but are not readily conducive to examining any effects that are progressive or delayed in nature.

Lack of Homeostatic Mechanisms. Using *in vitro* systems provides the researcher with the ability to study a discrete nervous system area isolated from normal *in vivo* homeostatic mechanisms. The use of *in vitro* techniques allows the exact and highly specific measurement of many fundamental biological processes isolated from other areas and from complicating factors contributed by the whole organism. *In vitro* systems offer much in the way of assessing mechanistic questions in a defined controlled system; however, it is an artificial system, and the effects seen *in vitro* must be confirmed or substantiated *in vivo*. The lack of homeostatic influences, such as the regulatory control of the neuroendocrine system, nutritional support provided by the blood circulation, interactions with adjacent cells, and the intercellular components that create a unique microenvironment, may limit the extrapolation of *in vitro* results to *in vivo* situations.

In the whole animal, chemical disposition is influenced by several factors. The presence of an intact blood–brain barrier is a major factor in determining a chemical's access to the brain. The absence of this barrier in culture conditions can be viewed in two ways. It may be advantageous in that the culture condition permits direct contact of a chemical with the cell, allowing assessment of the intrinsic toxicity of the chemical. The lack of a blood–brain barrier in culture, however, can also limit the extrapolation of *in vitro* results to the *in vivo* condition. Although *in vitro* conditions allow a chemical direct contact with nervous system cells, the lack of the *in vivo* filtering processes associated with the blood–brain barrier or lipid–aqueous partitioning (*26*) contributes to the artificial nature of the culture exposure. Therefore, although cell culture allows a precise determination of the chemical delivered to cells, it also poses a difficulty in defining toxicologically relevant doses. It must be noted that the blood–brain barrier is incomplete during prenatal stages of development and in specific regions of the nervous system. In addition, the permeability of the blood–brain or blood–nerve barrier may be compromised as a result of chemical exposure to either the test compound or by induction of other modulating factors. This would allow passage of components normally excluded from the nervous system.

Multiple Species Comparisons. *In vitro* systems offer the opportunity for the investigator to examine similar cell types from multiple species. One can measure responses in cells from various species easily when cell lines are used. The response of human cells can be compared with that of other species to address questions of interspecies selective toxicity. However, comparisons may be limited by the inherent differences in the origin of the cell line that are totally unrelated to species origin. Primary cell cultures are not readily available from human tissue. Human lines are derived from a limited number of progenitor cells. However, the development of new cell lines offers a wider variety of human cells to examine. For example a self-replicating culture HCN-1Aa was recently developed from human cerebral cortical neurons obtained from a patient with unilateral megalencephaly, a developmental neuropathology in which immature neurons continue to proliferate (*27*).

Heterogeneity of the Nervous System. *In vivo,* the development and differentiation of the nervous system depend on the interaction between neuronal and non-neuronal cell types. In a neurotoxic response, not all compounds directly interact with individual cells but may cause damage by indirect effects such as modifications of cell–cell interactions. As mentioned earlier, what has been perceived as a major advantage of primary cell cultures is the ability to obtain a culture enriched in one particular cell type. Such an isolated condition is an alien state for neural cells. Much work has focused on obtaining enriched cultures representative of neuronal populations in the brain, spinal cord, and ganglion. With these efforts it has been demonstrated that rarely does a neuronal culture exist in the absence of some glial cells and that dynamic interactions exist between the cell types in culture (*28,29*). In fact, in many cases neurons survive poorly in the absence of astroglial cells (*30*). When early postnatal cerebellar astroglial cells are cultured in the absence of neurons, they show a flat, undifferentiated morphology and proliferate rapidly. The addition of neurons to such cultures rapidly arrests glial cell growth and induce glial morphological differentiation into profiles resembling cerebellar astroglia seen *in vivo* (*31*). Similar effects are seen in cells isolated from other regions of the brain. Neuronal inhibition of glial cell division requires a neuron:glial cell ratio of at least 4 to 1. If the system falls below that ratio, many of the glial cells remain free of neurons and continue to proliferate, suggesting the need for membrane–membrane interactions. It has been reported that in culture the inhibition of glial cell proliferation by neurons can be modulated by the availability and type of serum in the culture media (*32*). Similar effects are seen with various astrocytoma cell lines (*33*). Astrocytomas pare among the most devastating CNS tumors, ranging from the slowly growing astrocytomas to the rapidly growing glioma multiformae. Cell lines from these tumors include rat C6, mouse G26-24, and human U-251, HTB-16, and A-172 cells, among others. Neurons plated on these astrocytoma cells rapidly bind to the astrocytoma cell and inhibit glial cell growth. A dynamic interaction between cell types has been reported with inhibition of glia-induced endothelial differentiation by lead

exposure in co-cultures of C6 glioma cells and retinal endothelial cells (*34*).

An example of cellular interdependency and influence on toxicity is the recent finding of the *in vitro* toxicity of trimethyltin (TMT), a chemical that produces specific neuronal necrosis in the hippocampus and astrocyte hypertrophy, and increased microglia staining. In a simple comparison of cell cytotoxicity, one finds distinct differences in dose levels required to produce a 50% increase in measurements of membrane permeability. Richter-Landsberg and Besser (*35*) studied the toxic effects of TMT on primary astrocytes isolated from 1- to 3-day-old neonatal rats and used neutral red assay to determine cell viability. They reported a dose of 2.5 μM TMT for half-maximal cytotoxicity in primary astrocytes treated for 24 hr. The toxicity of TMT on primary cultures derived from 1-day-old rat pups containing both astrocytes and neurons was higher with a half-maximal cytotoxicity in primary glial cells of 5 μM as measured by ethidium–calcein viability assay and by immunofluorescence (*36*). A third study examining the toxicity of TMT on a mixed glial population in culture (*37*) reported approximately a 30% decrease in cell viability at 10 μM TMT within 24 hr as measured by neutral red assay and lactate dehydrogenase (LDH) release. Given the steep nature of the dose–response curve for lethality *in vivo*, these differences *in vitro* raise concern. Although each study derived cells from rat pups within the designated time of postnatal day 0 to 2, there were several differences in the conditions of the culture. In the Richter-Landsberg and Besser study (*35*), the standard protocol for isolation of rat brain glial (*38*) was followed, which required a defined region of the cortex to be used for isolation. The initial cell preparation was grown for 2 weeks in flasks in serum-containing medium, followed by shaking to remove both microglia and oligodendroglia. The remaining cells were replated in 24-well culture dishes and used for toxicity studies within 2 days. In the study by Maier et al. (*37*), mixed glial prepared from postnatal day 2 rat pups by the method of McCarthy and de Vellis (*38*) were allowed to grow in tissue culture flasks for 2 weeks in medium containing fetal calf serum. They were then replated into 24-well culture dishes in which they were allowed to mature until 14 days *in vitro*. This 14- to 21-day period following replating is consistent with all neurobiological studies conducted on astrocytes and can be critical to the maturation state of the cells and the proportion of cells differentiated into oligodendroglia, astrocytes, and microglia. Thus, the difference in sensitivity could be due to the cellular population within each culture and the interactions between each cell type, the brain region from which the cells were derived, the maturation state of the cells at the time of toxicant exposure, the substratum on which the cells were plated, and the presence of serum.

During development, neurons and glial cells may interact by influencing differentiation. The anatomical organization of astrocytes and their ability to take up and metabolize synaptic transmitters suggest multiple functions for astrocytes in synaptic transmission (*39–41*). Glial cells extraneuronally regulate neurotransmitter concentration, thus potentially influencing the neurons (*42–44*). Numerous studies on the glutamine–glutamate neuronal–glial cycle suggest that glial cells act as modulators of neuronal activity (*45*). Glial cells may play an important role in maintenance of neuronal glutamate metabolism in cells that use glutamate as a neurotransmitter. Based upon the cellular localization of glutamine synthetase in glial cells (*46,47*) and glutaminase in neurons, a cycle has been proposed in which glutamate released by the neuron is taken up into astrocytes by a high-affinity uptake system (*48,49*). Once in the astrocyte, glutamate is converted to glutamine by glutamine synthetase and released. It is then taken up by the neuron and reconverted by glutaminase into glutamate. Gamma-aminobutyric acid (GABA) is released from astrocytes upon glutamate receptor stimulation (*50*) and could inhibit neuronal signaling. In models of chemical-induced glutamate excitotoxicity, any examination of neuronal cells in the absence of glia would be problematic. Glial cells contain the enzyme monoamine oxidase B (*51–53*). Thus, any chemical requiring local metabolism in the brain for its toxicity would not be detected in cultures devoid of glial cells, although such a culture condition is highly unlikely even at a high level of neuronal purity.

The role for astrocytes in neurotoxic responses has been supported in primary cell culture studies. Astroglia accumulate lead from the culture medium and concentrate it to levels as high as 1000-fold above the extracellular level (*54*). Similar reports exist for sequestration of iron in astroglia (*55*). Quantification of intracellular metal levels by graphite furnace atomic absorption spectroscopy requires a minimum of 2 million cells per sample, thus limiting the number of samples available for analysis. Of interest are the observations that glia in mixed cultures containing other cell types respond differently to lead than either oligodendroglia or astrocytes in pure cultures (*56,57*).

Examples in Pharmacology and Toxicology

Practical uses of *in vitro* systems have been established for development of pharmaceutical agents. In general, pharmacological agents are designed to act by a specific mechanism at a receptor site. Depending on the target site involved, one can develop a specific *in vitro* system that will allow for testing of drug efficacy and potency. In addition, information acquired during drug design, site of action of the drug being tested, and any confounding sites of action that would change the drug potency could lead to additional testing in focused systems. This is a unique environment in which a drug is designed to work via a specific mechanism; because this information is known, *in vitro* tests can be designed to evaluate this specific mechanism. The use of *in vitro* techniques to identify a drug for development, refine a drug's action and detect problems with a drug such that developmental costs can be minimized are all worthwhile in an environment where a mechanism of action, the target cell type(s), and/or cell functions are generally known. However, *in vitro* tests are not generally amenable for assessing potential unexpected or unknown drug side effects and testing is conducted in animals prior to human clinical trials.

A well-characterized mechanism for neurotoxicity is excess release of the excitatory neurotransmitter glutamate. Glutamate excitotoxicity is mediated, in part, via activation of postsynaptic *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. Cells that possess these receptors are susceptible to excitotoxicity; excess receptor stimulation leads to prolonged influx of sodium and calcium. This influx triggers a cascade of events that ultimately leads to cell death (*58*). Several glutamate analogs such as domoic acid also produce an excitotoxic lesion. Domoic acid accumulation in shellfish has caused outbreaks of human amnesia via loss of hippocampal and neocortical neurons, which are sensitive to activation of the glutamate receptor.

Identification and understanding of this mechanism of excitotoxicity has been aided by the use of a variety of *in vitro* systems. Hippocampal slice preparations and

organotypic cultures have been used to study electrophysiologic, biochemical, and pharmacologic consequences of NMDA receptor activation. Primary dispersed hippocampal and neocortical cultures are also used for characterizing excitotoxicants. Finally, both *Xenopus* oocytes and several neuroblastoma-derived cell lines have been transfected with expression vectors directing production of neurotransmitter receptors. Expression of such receptors in cell lines allows additional characterization of receptor responses in a different cellular context. The key reason for success in studying the NMDA receptor *in vitro* was the clear identification of the mechanistic target for glutamate agonists and antagonists. Clearly, unknown chemicals can be evaluated for excitotoxic properties *in vitro* using glutamate receptor-bearing cells. However, other, nonglutamate-mediated toxicity may be missed.

The situation as presented for pharmaceutical agents does not always exist in the development of chemicals used in agriculture or industry. One exception to this statement is certain insecticides designed to act at ion channels in the nerve membrane. The pyrethroid insecticides provide an example of pesticides which are neurotoxic but not cytotoxic. There is a reasonable correlation between increased transmembrane sodium flux and neurotoxicity, but pyrethroids have little or no effect on neuronal viability (*59*). Another successful application of an *in vitro* or *ex vivo* test is the prediction of the delayed neurotoxic potential of certain organophosphorous pesticides and industrial chemicals by measurement of neuropathy target esterase (NTE). The ratio of NTE aging to acetylcholinesterase (AChE) inhibition can be measured in normal brain homogenates or in cell cultures. This provides a prediction of the relative acute toxic and delayed neurotoxic potential of these agents. An illustration of the importance of bioactivation is provided by the organophosphates. The sulfur form (e.g., parathion) has very little toxic potential until bioactivated to the oxon form (e.g., paraoxon). This capacity of bioactivation is lacking in the absence of *in vivo* mechanisms and therefore remains undetected in a metabolically incompetent *ex vivo* or *in vitro* system.

Specific *in Vitro* Conditions and Techniques

In Vitro Technology

To conduct reliable *in vitro* studies, one must provide and adhere to certain specific requirements to ensure healthy reproducible neural cell cultures. One major requirement that distinguishes *in vitro* techniques from other laboratory methods is the need to maintain asepsis. Aseptic functions that occur in the cell culture laboratory include culture preparation, incubation, and handling. The cell culture laboratory should have access to sterilization methods appropriate for media, instruments, and biological wastes. The use of laminar flow cabinets with a continuous movement of sterile air over the work area provides a relatively low-cost environment under which cultures may be collected and manipulated. Further control of the cell culture environment (temperature, oxygen tension, carbon dioxide content) is critical and is generally provided by the use of cell culture incubators with uninterrupted power supplies. Most cell culture laboratories have access to refrigerated storage (4 to –20°C) capable of storing media and other reagents, as well as liquid nitrogen freezers for long-term storage of neural cell lines. An inverted phase-contrast microscope or, in the case of glial cells, a microscope with three-dimensional optics such as Hoffman or Nomarski, is vital to allow morphological assessment of neural cultures. Access to specialty chemicals, sterile pipettes, glassware, and cell culture vessels is also critical for a cell culture laboratory. Other equipment generally required includes refrigerated centrifuges, cell counters including hemocytometers, and pipetting equipment for the addition of media and removal of spent medium.

Culture Conditions and Cell Characterization

It has been well established that in any culture system the cellular phenotype is influenced by culture conditions. *In vivo* growth and differentiation of cells is dependent upon a complex set of environmental influences that include nutritional and hormonal factors, the nature of the substratum to which the cells adhere, and cell–cell interactions. Attempts have been made to mimic the *in vivo* condition with improvement and diversification of culture media and substrata. However, to produce a cell in culture that is identical to its *in vivo* counterpart, all external influences must be adequately mimicked.

Cellular Heterogeneity. In primary cultures the initial cell population is extremely heterogeneous, and it is impossible to obtain a group of cells in culture that is completely representative of those *in vivo*. For some specific questions, it may be desirable to have cultures of only one cell type. These cultures can be obtained from distinct brain regions by selection of donor optimal age. Once growing in culture, a cell type of interest can be selected by a variety of methods. For example, a cytotoxic antiserum may be used to kill one class of cells; deletion of a critical hormone could selectively eliminate a cell type. Glia and fibroblasts are frequently decreased by the use of DNA synthesis inhibitors that kill only dividing cells. Other methods, such as those used for glial cells, include manipulation of serum concentration in the media, and differential cell adhesion. All of these methods will aid in the purification process; however, no method has been found to ensure the absolute cell purity of the culture. The ability to provide a cell type-enriched culture offers a primary advantage over dissociated cell cultures. This offers the possibility to make biochemical measurements on large numbers of cells rather than on a single cell. Whether the results obtained from these pure cell cultures will be reflective of the *in vivo* condition is uncertain.

As growth conditions often may select for some phenotypes versus others, it is essential that the conditions of dissociation and culturing be identical for each experiment to ensure reproducibility. With heterogeneous CNS tissue, it is unlikely that the final cell population will reflect the initial population. The composition of a culture can change drastically with time if some of the cells in the population continue to divide. The more rapidly dividing cells will have a selective advantage over the nondividing cells. If one is using cloned cells from an original culture, the clones are limited in the number of times they can divide. Thus it is necessary to periodically isolate new clones. Phenotypic drift often occurs and mutant cells are difficult to identify in primary cultures.

Serum versus Chemically Defined Medium. Currently, most cultured cells are kept in media containing serum plus a basic nutrient mixture of required sugars, salts, amino acids, and vitamins. Serum contains additional growth hormones and growth and attachment factors. Attempts to fully mimic these components have been difficult. Chemically defined serum-free media have been devised for many cell lines and primary cell cultures. These media may contain salts, vitamins, amino acids, growth factors, hormones, and adhesion molecules specific to each type of cultured cell, and do not necessarily generalize to

other cells (*60*). Chemically defined media conditions have tempered the troublesome, uncontrollable, and undefined nature of sera, as well as problems of standardization and reproducibility of results with various batches of sera. However, it can be assumed that any extraneous supplementation will be minimal compared to the contribution of various components available *in vivo*. Because of various developmental factors present in the nervous system and limited knowledge of extracellular influences, fully defined media is very different from the *in vivo* physiological milieu of the cell.

Given the variety of media and supplements, one needs to be aware of the extreme variations in cellular metabolism and gene expression that can result under different media conditions. For example, the metabolism of epidermal growth factor (EGF) varies as a function of serum. EGF remains on the membrane surface of cells grown in a defined serum-free medium factor but is internalized in the presence of serum. Synthesis of glial fibrillary acidic protein (GFAP) and microtubule-associated protein by neural cells is influenced by both serum concentration and animal source. Adrenal cortical cells grown in medium supplemented with horse serum secrete large amounts of corticosterone but display a different morphology in fetal calf serum and produce only 1% of the steroid as made by cells in horse serum. The phenotype cannot be reversed by manipulation of serum type in the culture medium.

Cell Substratum. The substratum on which cells are cultivated contributes greatly to their metabolism and division. Frequently collagen, fibronectin, poly-D-lysine, polyornithine, and highly sulfonated tissue culture plastic are used as cell substrata. Some cells are anchorage dependent (i.e., require an adhesive surface on which to adhere) whereas others proliferate/differentiate either loosely attached, or in suspension as reaggregates. If cells are grown under conditions in which their state of adhesion is experimentally varied, many biochemical characteristics of the cells also change. For example, poly-D-lysine is used often in neural cell cultures, to increase cell–substratum adhesion. Many times poly-D-lysine is used to assure the continued attachment of cells throughout the washes required in various experiments such as precursor uptake studies, enzyme assays, or binding of isotopically labeled ligands to cell-surface receptors. Even for cell viability studies of dye uptake, maximizing the attachment of viable cells to the substratum is critical to experimental outcome. Although poly-D-lysine is often used, it can cause a number of phenotypic changes in cells, including changes in morphology, ultrastructure, and metabolism. Because cell–poly-D-lysine interaction can alter cAMP synthesis and interfere with normal ion channel functioning, this substratum should not be used with cells for electrophysiological experimentation. These effects are likely due to a direct and global action of the polycation on the cell membrane rather than to any specific modifications of the particular phenotypic trait being examined.

With cells that can be grown either in suspension or attached to a surface, differences in metabolism can be observed. In fibroblasts, various amino acid transport systems are used, depending on whether the cells are attached or suspended. Fibroblasts that are grown attached to a surface contain 5 times as much of the protein tubulin as cells grown in suspension. Substrate-attached fibroblasts are also more sensitive to cytolysis by antisera against the cell surface, although the actual number of cell-surface antigens is the same in both culture conditions. The substratum can regulate cell morphology and division rate. Fibroblast-like cells grown on culture plastic are flat and elongated and divide rapidly. The same cells grown on hydrophobic bacteriological plastic do not attach, are round in shape, and divide very slowly if at all. Experiments conducted on this regulation of division and morphology demonstrated that differences in the cells were not minor; fibroblast-like cells can initiate DNA synthesis only when they are sufficiently flattened on the substratum.

Cell Feeding. During the routine replacement of culture medium or "feeding," problems can arise from inconsistent cell culture techniques. Cellular phenotype can be altered in some cells by the addition of new culture medium or the manipulation associated with medium replacement. Therefore, care must be taken to insure that experimental effects can be distinguished from those caused by the addition of new medium. For example, with the addition of new medium, endothelial cells decrease plasminogen activator activity. This appears to be due to an inhibitory factor in the serum of the medium. It has been proposed that this effect can be circumvented by the use of chemically defined, serum-free culture media; however, with neural cells, addition of new growth factors in added medium or dilution of a released growth compound by media exchange may also present a transient change in the cell phenotype. As a result of culture medium replacement, the pH of the medium may rise transiently above 7.4. The processes induced at this pH are somewhat similar to those induced by growth factors. Partial replacement or methods to limit the need for medium replacement can partially overcome these problems.

Oxygen Tension. Atmospheric oxygen tension is a critical component that must be maintained for the culturing of nerve cells. In the majority of published protocols, the atmosphere of the cell within an incubator is maintained at a ratio of 95% air to 5% carbon dioxide. The viability of cerebral cortical neurons and the proliferation and maturation of astroglia cells is maximized at 10% partial pressure of oxygen (*61*).

Cell–Cell Interactions. Because of differences in cell–cell interactions, differences in metabolism and degree of differentiation can be observed between cells grown as reaggregates or attached to a surface. A simple example is the dramatic alteration in cellular physiology depending on the aggregation state of cells. An example of cell–cell contact in the regulation of hormone receptor function has been demonstrated in neural retina cells maintained in reaggregate cultures. The cytoplasmic cortisol receptor was decreased and the enzyme glutamine synthetase was not inducible by cortisol. However, in reaggregate cells the cortisol receptors are detectable and the glutamine synthetase is inducible at levels similar to *in vivo*.

Cell Cycle. The overall rate of protein synthesis is similar between dividing and stationary-phase cells; however, the rate of synthesis for about 40% of specific proteins varies as a function of cell phase. Therefore, experimental protocols involving reagents which alter cell cycle (e.g., antimitotic drugs) must be designed to distinguish between the primary experimental effect and the secondary consequences of altering the growth cycles of the cultures. Cell phenotypic changes are also associated with the cell cycle. Cells vary not only among different growth phases of the culture but also throughout the cell cycle. Under normal proliferative conditions, macromolecular synthesis in exponentially dividing cells is always quantitatively different from that in stationary-phase cells.

Age. The donor animal and duration of the culture period are both critical parameters in many organotypic, reaggregate, and dissociated primary cultures. Most attempts at primary culture of nervous tissue have

involved the use of fetal or neonatal material because of the difficulties in initiating cultures from the adult vertebrate CNS. These difficulties arise from the destruction of the neurites during cell isolation/preparation procedures and removal of cells from the projection areas. Because of differences in timing of development in various brain regions, neuronal cultures from each brain region have been successful only within a distinct identified age period. Even within the same brain region, individual cell types require distinct donor ages. For example, from rat brain cerebellum, granule cells are isolated from postnatal day 6 to 8 brain, but successful Purkinje cell cultures require isolation from fetal tissue. Glial cell cultures offer a broader window of age from which successful cultures can be obtained. Greater success has been achieved in culturing components of the PNS where primary cultures may be maintained from adult and even aged donors (*62,63*).

Neuronal cultures differ dramatically in the composition of glial cells, the maturation of the glia, the proportion of glial cell types, and the biochemical responsiveness of the culture. Although glial cultures can be generated from different ages, it is critical to the interpretation of experimental data that the donor age be maintained within a well-characterized biologic and morphologic window. For cortical astrocytes and oligodendroglia, this is between birth and 2 days postnatal. For each of these cell types, the culture age is critically important with regard to the responsiveness of the culture to manipulation. Although it has been claimed that primary nerve cultures can be maintained for several months by using supplemented nutrient media and a substrate that supports adhesion, it is well known that the metabolic properties of the cells will change over time. Factors of donor age, as well as age of the culture, must be addressed whenever primary culture systems are used to determine pharmacological or toxicological responses to various agents.

Slice cultures are prepared from infant animals with the age of the donor is a critical parameter in the degree of organotypic organization achieved in the final preparation. Usually, tissue is obtained from rats within the first week of life. By that time, a fair degree of tissue-specific cytoarchitecture has already been established and the peak of neuronal migration has passed. Within 2 to 3 weeks in culture, an original slice will thin to form a pseudomonolayer of cells and the degree of maturation and differentiation can be determined prior to experimental manipulation. For *ex vivo* studies, tissue can be obtained from any age animal; however, the maturation of the region of interest prior to sampling and interpretation of data must be considered.

Continuous cell lines are usually derived from tumorigenic tissue and can be obtained from donors of various ages. In culture, these types of cells have a useful life span of approximately 50 divisions characterized by progressively longer time periods between population doubling. Immortalized cell lines are generated from cell lines of limited life span that undergo crisis after which their growth potential changes and the life span becomes unlimited. Clonal primary cultures are derived from the progeny of a single cell, but the cells are limited in the number of divisions they can undergo. Once the cell line phenotype is established, it does not change; however, with increasing passages it slowly drifts with regard to physiological responsiveness.

The sensitivity of any culture to toxicants can be influenced by the age of the culture. Whether it is the influence of passage number in continuous cell lines, maturation and establishment of connections in a reaggregate culture, or the cellular maturation of primary cell cultures, each culture model requires that any experimental manipulation be conducted at an optimal and consistent age in culture. For example, the toxicity of MPTP to dopaminergic neurons is significantly greater in fully developed dopaminergic neurons and toxicity that is dependent upon ion channel activity will require the maturation of this physiological process for detection and examination.

State of Differentiation. Since many of the sources of tissue for primary cultures are from fetal or early postnatal animals, features of adult differentiated neurons or glial cells may not be present. Examples include cerebellar granule cells and dentate gyrus neurons; both cease division postnatally and do not express ion channels until several days in culture. In neuroblastoma and related cell lines, trophic growth factors alter the state of morphologic differentiation and overall gene expression. Well-characterized examples include nerve growth factor (NGF)-induced differentiation of rat pheochromocytoma (PC-12) cells and retinoic acid-induced differentiation of human neuroblastoma cells (SMS-KCNR; SNSY5Y). In both instances, treated cells extend complex neurites, express new neuronal cytoskeletal elements, and show prominent shifts in gene expression and slowed or arrested cell division. The state of differentiation can clearly affect expression of potential targets of neurotoxicity.

Characterization. In specific cases, one requires a relatively pure culture of neuronal or glial cells. To determine the purity of a culture, one must examine the culture not only microscopically for structural features but also by immunocytochemical methods to identify individual cells contributing to the culture. A problem central to all culture systems is cellular impurities and culture heterogeneity. It is, therefore, essential that both the characteristic morphology and antigenic phenotype of the cultured cells be characterized to permit the unique identification of these cell types. These may include either immunocytochemical or enzymatic markers that are associated with the plathora of CNS cells (Table 2).

One possible contaminating cell in any primary cell culture is the fibroblast. Attempts are made to eliminate this cell type from neural cultures by removing all brain meninges prior to dissociation, preplating for early adherence removal, and adding cytotoxic agents for proliferating cells. The case is similar for glial cells as a contaminating factor in neuronal cultures; however, detection of all types of glial cells requires the use of a number of antibody evaluations. One of the major concerns with contributing glial cells is the surface area of the glia relative to neurons. Although one may determine by counting number of cells that glia contribute less than 5% of the culture, each one of these cells has the surface area of hundreds of neurons. With glial cultures, the use of differential adherence or gradient fractionation aids in the generation of enriched cultures of individual glial cells. However, recent evidence suggests that factors previously contributed by astrocytes are the product of contaminating microglia cells within the culture preparation. As little as 1% contribution of microglia can significantly influence the interpretation of data derived from astrocyte cell cultures. Not only must the culture system be characterized in use with regard to contributing cell types, but the maturation state of the specific cell type of interest must be determined. For example, during maturation, glial cells—both astrocytes and oligodendroglia—will progress through distinct periods of surface antigen expression, and the use of the standard antibody staining to GFAP may not detect all contributing astrocytes.

Table 2. Cell types and immunohistochemical markers.

Cells	Markers
Astrocytes	Glial fibrillary acidic protein S-100 α,α-Enolase Glutamine synthetase A2B5 G_{D3} G_{Qib} Glutathione *S*-transferase Pyruvate carboxylase Vimentin Laminin
Oligodendrocytes	Myelin basic protein Myelin-associated glycoprotein Glycerol phosphate dehydrogenase GM4 (sialogalactocylceramide) Galactosylglycerides Sulfogalactosylceramides Sulfogalactosylcerides 2′,3′-Cyclic nucleotide 3″-Phosphohydrolase Neurite growth inhibitors 35 and 250 Wolfgram protein Proteolipid proteins Transferrin Biotin Cholesterolester hydrolase Carbonic anhydrase II
Endothelial cell	Factor VIII Alkaline phosphatase
Fibroblasts	Fibronectin
Ependymal cells	Beating cilia A2B5
Neurons	Neuron-specific enolase Tetanus toxin-binding gangliosides
Microglia	OX-42 Lectin–B_4 *Griffonia simplicifolia*

Surveillance. Antibiotics and/or fungicides often are added to a cell culture preparation to prevent contamination. Such additions can reduce cellular activity and modify drug-induced neurotoxic effects. Unlike bacteria and fungi, contamination of mycoplasma usually does not result in turbid growth or macroscopic alterations of the cells. However, mycoplasma contamination may cause adverse effects such as changes in metabolism, growth, viability, DNA, RNA, protein synthesis, morphology, and virus propagation, thus leading to unreliable experimental data. For example, in a mixed glial preparation containing microglia, any contaminating factor will activate the microglia into phagocytic activity. The source of contamination may originate from initial culturing techniques and in the case of a cell line can be propagated among a number of research laboratories. As a quality control measure, all culture systems should be evaluated for the presence of any bacterial, fungal, or mycoplasmic contamination prior to use.

Other Culture Condition Factors. There are a number of other factors unique to culture systems that raise concern for neurotoxicologists. For example, the viability and plating efficiency (number of cells that attach to the substratum with initial plating) of individual cells is greatly influenced by the technical manipulation of the cell source. For primary cultures, the method of dissociation has a major influence. Enzymatically dissociated cells generally survive better than those dissociated by mechanical means. It has been suggested that at early prenatal stages a mechanical disruption of cells produces greater cell survival and enzymatic disruption produces greater survival of cells isolated from late prenatal and postnatal ages. For cell lines, the storage condition of the cells (e.g., frozen in DMSO or glycine, by quick or slow method, in media or buffer) and methods of plating can influence the proportion of cell viability. For each culture system, these factors can influence greatly the ability to compare experimental data across various laboratories using different techniques or different cell sources.

In summary, culture systems offer the potential advantage of examining a direct cellular response to a chemical, determining biological processes at a level that would not be accessible *in vivo*, and assessing underlying mechanisms associated with cellular development and functioning of the nervous system. Although chemicals can be easily added and withdrawn from the cultures, and their effects directly probed in culture systems, caution should be used when correlating effects occurring *in vitro* to those observed in the intact animal, where additive interactions are likely to occur. One must remember several concepts: *a*) A number of different, sometimes competing, processes influence the ability of a toxin to attack and destroy specific cells. Metabolism of the administered agent by a nontarget cell or tissue may be responsible for bioactivation or detoxification of the compound or its metabolite, affecting the vulnerability of the cells to the neurotoxin. *b*) A cell culture is simpler and much more homogeneous than any tissue, in particular the CNS. Removal of many cell types and barriers can facilitate diffusion or even active transport of the compound or its metabolite, limiting or enhancing toxicity by determining at which sites the toxin can reach sufficiently high concentrations to interfere with vital cellular processes. *c*) The capacity of the cell to repair or replace damaged organelles or enzymes can also be critical in determining cell survival after toxic insult and may depend on neighboring cells and physical barriers, which would be absent in the culture.

Although culture systems are relatively convenient to use, their ability to predict the neurotoxicity of a chemical *in situ* or the potency of a series of structurally related analogs depends on whether the culture system expresses the target for a given neurotoxic effect. Any culture system derived from resident cells of the nervous system represents an artificial system with regard to the *in situ* condition. Therefore, any experimental use of such systems to address specific scientific questions requires that precautions are taken to ensure biological relevance, validity and reliability of the data. Data generated from *in vitro* studies must be interpreted within the limits and framework of the experimental design. *In vitro* studies are relevant only if the target site is contained within the culture model. Any extrapolation to the *in vivo* condition must take into consideration modulating factors that are present in the whole animal, e.g., blood–brain barrier, lipid partitioning, metabolic activation, or detoxification of a chemical.

Model *in Vitro* Systems

Introduction. All cell culture systems represent cells that are no longer part of an integrated neural network and may develop an altered appearance, metabolism, and an altered response to test chemicals (Figure 1). There are numerous vertebrate primary culture systems representing a specific region or cellular component of the nervous system. Historically, the first organotypic cell culture system was based on fragments of nervous tissue from frog embryo placed in a drop of clotted lymph tissue. This system demonstrated that each nerve axon or dendrite is an extension of the neuronal cell body. These studies became the prototype of the explant cell culture technique. The second technique, dissociated cell culture, was first introduced in the 1960s. In this method, the tissue is dissociated mechanically or enzymatically, resulting in a suspension of dispersed cells. These cells are then cultured on substratum in a culture dish. A third system, reaggregate cell culture, was initiated to provide a tissuelike environment for the cells. In this method, the dissociated cells are placed in a rotating flask and small round aggregates are found floating in the media. A fourth system is

that of clonal cell culture lines, which is established by culturing the progeny of a single cell. Neural cell lines come from endogenous tumors or from chemically or viral transformed cells; tumorigenic properties of these cell lines present obvious limitations to their usefulness. Efforts to use human cell lines within neurotoxicology have been limited by the atypical nature of the cells, for example, their expression of high levels of glutathione (*20*).

Uses and Limitations. For examination of altered nervous system functioning, isolated models have been selected that contain key biochemical and morphological features which are specifically targeted by neurotoxicants *in vivo*. Each type of tissue culture system to be discussed has its own specific advantages and disadvantages (Table 3). Dissociated cell cultures allow for visualization of individual living cells and for monitoring both morphological and electrophysiological features. Although the histotypic tissue organization is lost as a result of the dissociation procedure, it is believed that neuronal and glial cells migrate to rearrange themselves on the substratum and differentiate according to their function and abilities. However, *in vivo*-like structures cannot be obtained by this technique. Dissociated cell cultures are more accessible to experimental manipulation than slice cultures and are easier to obtain and maintain. It is possible to obtain and correlate biochemical, morphological, electrophysiological, and molecular data from a single cell. Additional purification methods can be used to enrich a particular cell type in a dissociated cell preparation, but it is difficult to obtain and define pure cultures of any one cell type.

Reaggregate cultures offer a more structured, three-dimensional extracellular space that more closely approximates the *in vivo* conditions for cell growth and development. Initially, the cells inside an aggregate are distributed in a random fashion followed by reorganization into a pattern formation similar to the *in vivo* structure. These preparations emphasize the importance of cell contact and histotypic organization by identifying markers induced in specific cells similar to *in vivo* yet absent in dissociated cell cultures (e.g., glucocorticoid-induced glutamine synthetase in retinal glial Muller cells). In hopes of similar biological advantages, some laboratories use three-dimensional matrix cultures made from components such as collagen and fibronectin. Clonal cell lines of tumoral origin provide homogeneous cell populations in large quantities in a very reproducible manner. Usually the common cell lines of choice are those that continue to express in culture the differentiated properties of their normal cell counterpart.

It must be noted again that these cell culture systems represent cells that are no longer part of an integrated neural network and may develop an altered appearance, metabolism, and an altered response to chemicals. Some believe that these concerns can be addressed by experimental design and choice of end points. No such efforts, however, will compensate for the isolation of a system from the natural neural environment and all of the systemic influences on that environment. The ability of culture systems to predict the neurotoxicity of a chemical *in situ* or the potency of a series of structurally related analogs can depend on whether they express the target for a neurotoxicant's action.

Types of Culture Systems Used in Neurotoxicology. ORGAN CULTURES. Large parts of the spinal cord, ganglia, or sensory organs (e.g., eye) can be maintained as organ cultures. Other neural organs are difficult to perfuse in a physiological manner, if at all. An example of an organ that has been studied using tissue culture is the eye. In these experiments, the eye is isolated under anesthesia and cannulated at the ophthalmociliary artery (*64,65*). A test substance can be introduced or washed out by the perfusion solution via the cannula. Effects caused by the administration of a chemical are usually measured by electrophysiological techniques. In the eye the reference electrodes are positioned at the

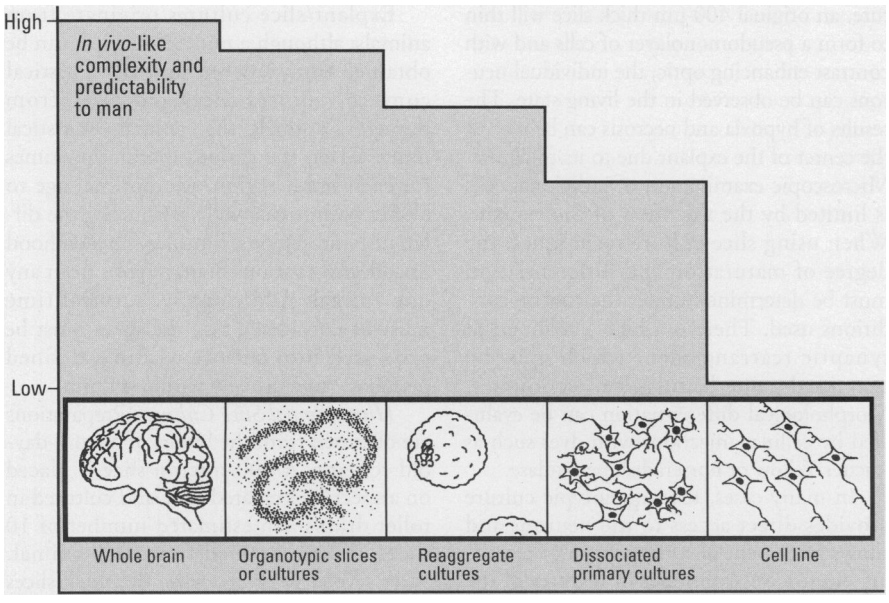

Figure 1. Model culture systems.

Table 3. Cell culture conditions.

Cell dissociation technique
Mechanical vs enzymatic
Source and original donor
Species - rat, mouse, human
Passage - clonal cell line
Age - primary cultures
Medium supplements
Growth factors
Proteins
Nutrients
Medium contaminants
Bacterial
Fungal
Mycoplasmic
Serum vs chemically defined medium
Sera source - bovine, horse, calf, fetal
Defined medium - identification for individual cell types
Cell feeding schedule
Cell substratum
Glass
Plastic
Collagen
Fibronectin
Polylysine
Matri-gel
Cell phase
Dividing cells
Stationary cells
Cell growth
Cell differentiation
Cell age
Developing
Mature

cornea and at the posterior pole. Standing potential, electroretinogram, and light peak recordings are made using a glass pipette placed in the vitreous humor.

It is assumed that the concentration of a test substance at the target cells is comparable with the *in vivo* situation if the concentration in the perfusate is the same as that in the bloodstream. A further advantage is that electrophysiological effects can be measured without knowing the target cell type. The disadvantage is that for any individual organ, recordings can be made for only a limited period of time. In the case of the eye this time period is a maximum of 6 hr. Therefore, only acute effects can be measured. It is not possible to determine the reversibility of the induced effects beyond this limited time period.

EXPLANT/SLICE CULTURES. Organotypic cultures are derived from explants of relatively undifferentiated embryonic brain, spinal cord, or sensory organs and develop into integrated neuronal and glial populations. For some regions of the nervous system, the age of animals used in explant cultures can vary from embryonic to postnatal periods but must remain constant across comparisons (*66*). The tissue explant may be a thin slice, a chunk, or the complete organ, as is the case for the sympathetic ganglia. The main advantage of this system is the presence of a three-dimensional organization in which some of the organ's structural and functional characteristics are retained. The organotypic explants/slices can survive in culture for a week to months, depending on the culture conditions. The disadvantage is the difficulty in quantifying small changes in differentiation or viability, depending on the parameters measured. The nutrient supply (e.g., oxygen, media compounds) available to the cells within the slice/explant is dependent on the diffusion rate and is governed by the thickness of the slice/explant. Often, depending on the thickness of the slices after prolonged culture periods, a hypoxia in the center of the tissue resulting in necrosis cannot be precluded. Only in a small layer of the tissue is it possible to maintain *in vivo*-like physiological conditions.

The goal of this culture technique is to obtain a preparation with a high degree of cellular maturation and differentiation and with an organotypic organization with the ability to assess individual neurons. The roller-tube technique first used with nervous system tissue has allowed preparations to be maintained *in vitro* for prolonged periods of time. These systems can be used to study processes involved in nervous system development such as specificity of axonal growth and the mechanisms involved in target contact. Organotypic slice cultures retain, at least for a limited period of time, important organizational features of the original tissue and allow for the morphological identification of specific cell types as well as the electrophysiological responses. Given the massive denervation and cell death caused by the explantation procedures and the novel influences of the tissue culture environments, it cannot be assumed that the properties of organotypic cultures are identical to their *in vivo* counterparts. What can be experimentally exploited, however, is any particular property of the cell that is the same under both *in vivo* and *in vitro* conditions. This culture technique has proven worthwhile in the use of sympathetic ganglia cultures to characterize and purify NGF and in the rat hemidiaphragm preparation to study reinnervation and rate of acetylcholine receptor (AChR) turnover. These cultures have been helpful in solving several problems that could not be addressed in animal experiments.

Slice cultures are prepared from infant animals; the age of the donor is a critical parameter with regard to the degree of organotypic organization achieved in the final preparation. Usually, tissue is obtained from rats within the first week of life. Within this time, a fair degree of tissue-specific cytoarchitecture has already been established and the peak of neuronal migration has passed. Within about 2 to 3 weeks in culture, an original 400-μm thick slice will thin to form a pseudomonolayer of cells and with contrast enhancing optic, the individual neurons can be observed in the living state. The results of hypoxia and necrosis can be seen at the center of the explant due to its thickness. Microscopic examination of individual cells is limited by the thickness of the explant. When using slice culture techniques, the degree of maturation and differentiation must be determined under the culture conditions used. There is a high likelihood of synaptic rearrangement which must be examined using histological techniques. Morphological differentiation can be evaluated by cellular injections with dyes such as Lucifer Yellow or horseradish peroxidase.

In many cases, the organotypic culture provides direct access to the neurons and allows placement of stimulating and recording electrodes. A number of features of the slice culture make it attractive for evaluating the electrophysiological activity of individual cells and determining the effects of various pharmacological agents. The presence of functional synapses needs to be assessed by electrophysiological recording techniques that require sophisticated methodologies and critical controls such as temperature. Both the excitatory and the inhibitory systems need to be evaluated. Receptor activity has been studied in various organotypic slice cultures. Such receptor studies include excitatory amino acids, GABA, acetylcholine, norepinephrine, opioid, and growth factor receptors. Pharmacological comparison of these receptors *in vivo* and in slice preparations show identical characteristics not only in activity but also in cellular distribution. This is not always the case for dissociated neurons.

Although slice cultures display a high degree of neuronal differentiation, this technique does not ensure that all cell types survive or that all important properties such as receptor sensitivity or neuronal connections are established. Although important features of the characteristic cellular and tissue organization of the original tissue are retained, the multitude of connections from various other brain regions is not represented. Loss of afferent input to the slices results in synaptic rearrangement within the slice culture. The absence of both efferent and afferent connections that would normally occur *in vivo* is a significant factor in determining the types of questions that could be addressed with such a system. This procedure demands from the experimenter an understanding of the morphological characteristics of the region examined.

Explant/slice cultures originate from animals; although a number of slices can be obtained from any one animal, statistical concern requires slices generated from numerous animals. (i.e., animal = statistical unit). Given the various maturation times for each brain region, the optimal age to obtain brain tissue will vary among the different brain regions, limiting the likelihood of collecting various brain regions from any one animal. Additionally, survival time must be considered i.e., the slices must be processed into culture within a defined period of time, usually within 45 min.

Hippocampal Slice Cultures. Preparations are obtained from the brains of 5- to 7-day-old rodents. A 400-micron slice is placed on a specially prepared slide and cultured in roller drums. An estimated number of 10 slices can be obtained from each animal. After several weeks *in vitro*, the thick slices will flatten to virtual monolayer thickness thus allowing observation of individual

neurons under phase contrast microscopy. This flattening is due to a lateral migration of nerve cells that are located in the deeper layered structures. This reorganization is proposed to be due to a limited oxygen supply at the deeper layers. Additionally, any structures adjacent to the cut surface or the severed afferent and efferent fibers degenerate. With the hippocampal slice, the reorganized three-dimensional structure retains the original hippocampal cytoarchitectural relations (*67*). Morphological differentiation can be maintained similar to *in situ* conditions for both the pyramidal and granule cells. Synaptic organization can also be maintained; however, aberrant supragranular projections are often reported. Techniques using Golgi impregnation and dye injections allow evaluation of morphological differentiation and dendritic arborization.

In slice culture, neurons and associated cells can be maintained for relatively long periods of time, thus allowing for extending periods of exposure to assess toxicological effects. Some researchers claim that slice cultures can be viable for up to a year; however, the cellular phenotype will change with age. Brain slices, especially the hippocampal slice, have been used extensively for neurobiological studies and the approaches have been adapted for utilization in neurotoxicity evaluation. For example, the hippocampus is vulnerable to excitotoxic agents such as kainic acid, which causes a neuronal necrosis of the pyramidal cells. The excitotoxic effect of kainic acid has also been demonstrated in the hippocampal slice culture, with a selective vulnerability of the CA3 pyramidal cells and no effect on the CA1 neurons, granule cells, and AChE-positive interneurons (*68*). This ability to detect a selective effect is critical in assessing neurotoxicity because *in vivo*, rarely are all cells of any one type affected. The basis for neuronal selectivity remains unknown. The hippocampal slice preparation has also been used in assessing the toxic response to bismuth. In agreement with human data, acute exposure had no effect (*32*). However, chronic application produced morphological evidence of pyramidal cell degeneration. The hippocampal slice preparation has been used to study the effects of organoalkyl metals such as trimethyltin and triethyltin.

In situ, the hippocampus is critically involved in the process of long-term potentiation as a component of synaptic plasticity. This process is of interest in many chemically induced alterations in neural functioning. The hippocampal slice preparation was evaluated electrophysiologically after exposure to the known hippocampal toxicant trimethyltin and showed similar results to *in vivo* effects (*69*); however, some important differences were noted. These researchers proposed the use of the hippocampal slice culture preparation as a screen for neurotoxicity which would be able to differentiate effects of chemicals on excitatory and inhibitory systems, mechanisms underlying neuronal plasticity, and regional differences in susceptibility to toxic insult. However, such uses for this test system are well outside the criteria set for a screening test and given the technical demands to confirm the integrity of the slice and the complexity of the hippocampal system, this test system would probably be more useful in addressing specific mechanism-based questions of both basic neurobiological and neurotoxicological nature.

Other Brain Slices. Other brain regions have been cultured successfully by the slice technique including the cerebellum, cortex, and the locus coeruleus. Like the hippocampal slice, the dendritic arborization of neurons derived from the locus coeruleus or cerebral cortex are similar to the *in vivo* structure. The cultured cerebellar Purkinje cells, however, form multipolar dendrites or dendrites of reduced complexity as compared to the *in vivo* state. Co-cultured slice cultures have been used to establish an *in vitro* analog of *in vivo* axonal connectivity among various brain regions. The ability to determine the success of fiber infiltration and connectivity is dependent upon the selectivity of immunostaining for a particular cell type. For example, in co-cultures of locus coeruleus and cerebellar or hippocampal slices, the invading fibers from the locus coeruleus can be visualized by immunohistochemical staining with antibodies to tyrosine hydroxylase. The co-culture technique has been used to study trophic factor interactions, axonal targeting, and synaptic transmission. It offers a unique method to examine such processes under the influence of various agents; however, the effectiveness of the model will reflect the mechanistic question asked.

PRIMARY CULTURES. *Suspension and Reaggregate Cultures.* Reaggregate cultures have great utility for examining biochemical end points where a large cell population is needed. Brain tissue is dissociated into a suspension of individual cells and incubated under gyratory conditions to form a confluent monocellular layer with patterns of cell alignment similar to those seen *in vivo*. Within 1 hr, small cell clumps (reaggregates) are formed. After 2 days these reaggregates have a stable diameter of 0.15 to 0.8 mm depending on the dissociation technique and culture conditions (*70–72*). Like explants/slices, a general disadvantage of reaggregates is the decreased supply of oxygen and other components of the culture medium to the center of the reaggregate. Dependent on the tissue from which the cells were derived, several layers can be discriminated with reaggregates with patterns of cell alignment similar to those seen *in vivo* (*73–75*). In addition, some foci of cells of the same cell type are observed after some days in culture innervating other parts of the reaggregates. The cells undergo morphological differentiation including synaptogenesis and myelination (*74,76,77*). In a developmentally regulated pattern, the cultures express cellular specific proteins for neurons and glia. Critical to this technique are the age of the donor, the nervous system site extracted, the culture conditions, and the dissociation procedure used. Not only are these features critical to the viability of the culture but also to the degree of cytoarchitectural development. Reaggregate cultures can be maintained by rotation under continuous incubation conditions using chemically defined media for prolonged periods of time (maximum reported > 6 months) (*72*). Thus, the cells can be used immediately in suspension to assess acute biochemical responses or used for future examination of biological responses. Reaggregates have been proposed for use in toxicological studies (*78,79*).

Dissociated Primary Cultures. The dissociated primary culture is the most widely used *in vitro* system in neurobiology. Typically, tissue is removed from the embryonic or fetal animal and dissociated mechanically or by proteolytic enzymes. The cells then are washed and placed in culture dishes. Usually, within an hour living cells will adhere to the culture dish substratum. Cells of the CNS are anchorage dependent and those that do not adhere usually die. The fraction of cells which adheres and survives varies greatly with the type of tissue. The plating efficiency is dependent on the dissociation technique, the type of substratum, the culture medium composition, and the type of tissue. A reduced initial survival rate may induce a change in the cell type composition, possibly resulting in an artificial enrichment or depletion of certain cell types. After the initial selection of cells surviving the dissociation process, there is a selection for cells compatible with the culture environment.

The presence and amount of serum and trophic factors, oxygen tension, the composition of the substratum, and seeding density strongly affects the viability and differentiation of cultured cells (dedifferentiation, transdifferentiation, differentiation inhibition, induction of differentiation). With CNS tissue, alterations in technical parameters such as age of donor, dissociation techniques, seeding density, and medium composition can greatly influence the characteristics of the culture.

Neuronal primary cultures. Much work has focused on obtaining cultures representative of neuronal populations in the brain, spinal cord, and ganglion. For each of these preparations, the age of the donor is critical for successful culturing of each cell type from different brain regions. Therefore, one is forced to maintain a consistency in donor age in order to generate a culture preparation. The dissection techniques and nutrient media are unique to each level of the neuroaxis and are empirically derived (*6,7*). For example, preparations of hippocampal neurons are derived from fetal brain tissue where cortical and cerebellar neurons can be derived from postnatal brain tissue. Even within a brain region such as the cerebellum, individual cell types require distinct donor ages. e.g., cerebellar granule cells are isolated from postnatal day 6 to 8 brains whereas successful Purkinje cell cultures require isolation from fetal tissue. Although dissociated cell culture techniques have been successful for numerous brain cell regions and cell types, cultures of dissociated peripheral neurons, sympathetic ganglion cells, and dorsal root ganglion cells have been a more recent addition. This is apparently due to their fastidiousness with respect to their culture media. However, successful conditions have been established and such cultures of the peripheral neurons have been used extensively to study problems of nerve differentiation and electrophysiology. As previously mentioned, whether these cultures will be reflective of the *in vivo* condition is a question that is continually asked. In many situations this is not the case. For example, Davenport et al. (*80*) were unable to detect in primary neuronal cultures of either the cortex or the cerebellum the critical *in vivo* distinction of differential sensitivity to monohalomethanes between cerebellar and cerebral cells.

Glial primary cultures. A number of techniques and media conditions exist for the generation of dissociated primary cell cultures of nervous system glia cells. Usually a mixed glial culture is derived from rat or mouse cortex between birth and postnatal day 2 (*38*). Between 6 and 18 pups contribute to each culture preparation comprised of three phenotypically distinct glial cells, the astrocyte, the oligodendrocyte, and the microglia. Based upon differential cell adhesion, each cell type can be subcultured to a relatively enriched population. Although much information is available on each individual cell type in culture, only recently have the interactions among the various glial cells been appreciated. In addition, results previously obtained from a pure culture of astrocytes must now be reconsidered in light of new information on the function and responsiveness of other glial cells and techniques used to assess culture purity.

Depending on the age of the donor, cell cultures differ dramatically in the glial cell composition, the maturation process, and biochemical responsiveness. Therefore, it is critical to any interpretation of experimental data that the donor age be maintained within a well-characterized biological and morphological window. For rodent cortical astrocytes and oligodendroglia, this is between birth and day 2 postnatal (*38*). Individual glial cell cultures can be generated based upon differential attachment of each cell type. Once a mixed glia culture has been established, e.g., in approximately 2 weeks, culture flasks can be shaken (250 rpm) to dislodge both microglia (within 2 hr) and oligodendroglia (18 hr) from the underlying astrocyte monolayer. A microglia culture can be obtained from these mixed glia cultures by plating of the media removed after 2 hr of shaking. Cells are allowed to attach to tissue culture plates over approximately 1 hr. The media is then removed and replaced with fresh media. After shaking, astrocytes can be subcultured by enzymatic detachment, filtration, and an initial plating period of 1 hr to remove any remaining contaminating microglia. Additional steps must be taken to ensure removal of any contaminating microglia from the oligodendroglia subculture. After cellular enrichment, medium conditions are manipulated with 1% fetal calf serum or chemically defined medium to ensure differentiation of progenitor cells to oligodendroglia. The generation of enriched subcultures depends upon a relatively large number of animals.

The majority of experiments using Schwann cell cultures have investigated the normal interactions between Schwann cell and axons. Three approaches have been used to isolate Schwann cells for culture. Fresh sciatic nerve tissue isolated from 2-day-old rat pups undergo enzymatic digestion with trypsin and collagenase, followed by trituration and plating for 24 hr. These cultures initially contain both bipolar spindle-shape Schwann cells and fibroblasts. The rapidly dividing fibroblasts are removed by the addition of the antimitotic agent cytosine arabinoside for 48 hr followed by removal for 6 hr and readministration for 24 hr (*81*). Cells can be be used for experimentation after being allowed to recover for an additional 24 hr in normal media. Typically, 0.8×10^6 cells/12 pups can be obtained, with approximately 95% characterized as Schwann cells. A similar procedure can be used to isolate Schwann cells from frozen neonatal sciatic nerves (*82*). Sciatic nerves from 2-day-old rat pups can be slow frozen in media, DMSO, and fetal calf serum (50%) and stored in liquid nitrogen. This method differs from the fresh tissue procedure in that frozen nerve cells require an additional 2 days to recover from the initial dissociation. Thus, this procedure requires a total of 7 days from dissociation to final use rather than the 5 days required when using fresh tissue. One major advantage to the use of frozen tissues is that tissue can be collected over time and stored until a sufficient amount is obtained for an adequate cultured cell yield. Schwann cells from both preparations appear identical in morphology and in their response to axonal membrane mitogens. An immortalized Schwann cell line has been produced and offers the advantage of rapidly generating extremely large numbers of Schwann cells that are genetically identical (*83*). These cells are similar to primary Schwann cells in their immunoreactivity to Ran-2 and glial fibrillary acidic protein antibodies and in their ability to segregate individual neurites in cultured dorsal root ganglia (*83*). When plated on the artificial basement membrane matrigel, the cells appear to react similarly to primary cells; however, their proliferation rate is decreased and the cells take on a spindle-shape morphology, suggesting a termination of proliferation and initiation of differentiation (*84*). Whether primary cells or cell lines, Schwann cells are very sensitive to the substratum and the serum concentration. They grow best on laminin or other extracellular matrix substratum and they require high levels of serum (10%). They do poorly in low serum (1%), especially in any studies examining a response to chemical exposure.

Data suggest that Schwann cell cultures can be used to study the initial proliferative responses and the transduction mechanisms involved in the proliferative response of Schwann cells to axonal membranes (*85–90*). Schwann cell expression of myelin-related proteins is exquisitely sensitive to axonal contact. In culture Schwann cells do not make a detectable amount of the myelin-specific protein P_0 (*81*). Early experiments examining toxicant-induced alterations in Schwann cell functioning focused on the effects of lead due to the *in vivo* effect of a demyelinative peripheral polyneuropathy. Pleasure et al. (*91*) demonstrated a lead-induced decrease in the rate of cell proliferation with no alteration in the ability to differentiate. However, in many cases of peripheral nerve toxicity, the chemical of interest produces a characteristic demyelination *in vivo*. Although it is possible to establish a co-culture with axons in which Schwann cells produce myelin, these procedures are extremely difficult and the degree of myelination is minimal, unlike the normal *in vivo* situation. While limited aspects of Schwann cell function can be examined *in vitro* any interpretation of data within the framework of *in vivo* toxicant-induced peripheral nerve demyelination is limited if not impossible.

CONTINUOUS CELL LINES. Continuous cell lines are transformed cells derived from neuroblastomas, gliomas, and pheochromocytomas with a useful life span of approximately 50 divisions. Cell lines of limited life span often undergo crisis after which their growth potential changes and their life span becomes unlimited. These cell lines are termed immortalized cell lines. The major attributes of continuous clonal cell lines are homogeneity and the ease with which a large quantity of cells can be grown. Features of established cell lines include the capacity to undergo an unlimited number of cell divisions, altered cell and colony morphology, lack of locomotion, lack of contact inhibition, lack of density-dependent inhibition of cell multiplication, loss of anchorage dependence, and high fibrinolytic activity (*92*). In addition, once the phenotype of the cell line is established it does not change. However, it has been observed quite often that the cells will drift with regard to physiological responsiveness with increased passages.

Cell lines are available for a number of donor species, but availability of neural cell lines is rather limited. Lines of cells exist that are representative of neurons (neuroblastomas) and glia (oligodendrocytes, Schwannoma, astrocytomas). Because of the numerous limitations of working with tumor-derived cells, cell lines devoid of tumoral properties have been generated with the use of retroviral vectors for transferring oncogenes to glial or neuronal progenitor cells in culture. With some rapidly dividing cell types, such as glia and myoblasts, a large cell population can be derived from the progeny of a single cell. It is therefore possible to have clonal primary cultures, which is the only way to assure that all of the cells in the culture are phenotypically identical. However, clonal primary cells are limited in the number of times that they can divide. This limited life span of cultured normal cells has been associated with *in vivo* aging. Many of these cell lines display properties of their normal cell counterpart. For example, the v-*myc* immortalized sympathoadrenal progenitor cell line, MAH, differentiates into neurons upon exposure to fibroblast growth factor. Neuroblastoma C1300 cells can be induced by serum modification to extend neurites.

One problem with the use of clonal cell lines is the limitation in the number of cell lines available that express well-characterized *in vivo* phenotypes. The derivation from neoplastic tissue suggests an origin which makes the cells abnormal and detracts from their usefulness. However, it needs to be mentioned that all cells acquire abnormal characteristics when placed in a tissue culture environment. Although continuous lines lack the type of growth regulation seen *in vivo*, the critical point for experimental use is whether the cells express the differentiated characteristic of interest identical to those *in vivo*. If they do, information on the binding characteristics of the test compound to the target receptor or binding site molecule may be studied as an important first step in the toxicity. However, caution should be exercised prior to using cell lines for toxicity studies even if they express the differentiated characteristic of interest. As the genetic amenability and response to a test compound may be dramatically changed in cell lines as compared to the original primary cells, unforeseen cell line specific effects may occur. Thus, the data may not be applicable for direct extrapolation to the *in vivo* condition.

One of the major limitations of continuous cell lines is the difficulty in inhibiting cell division experimentally to obtain a stable population of differentiated cells. This is in contrast to the *in vivo* situation where the end cell in a differentiation sequence usually does not divide. The cellular homogeneity of cell lines may be appropriate for the purpose of studying selectivity of neurotoxicants in toxicological studies. This homogeneity would limit the cellular interactions and the cells would not be exposed to the complex network of messages typical for neuronal circuits. In many cases, differentiation is induced by chemicals and drugs by a mechanism that is not fully understood. It is not known if it is comparable to the differentiation process that occurs *in vivo*. In general, cell lines show much less sensitivity to toxicants than primary cell cultures. For example, the cholinergic cell line NG-108-15 is much less sensitive to AF64A than primary cells (*93*).

Genetic stability of any given phenotype is critical to the reproducibility of experimental findings. Clonal cultures can undergo variant selection that can be explained somewhat in terms of point mutations, deletions, or chromosomal rearrangements. Additional data suggest that cells are able to respond in a variety of ways to strong selective agents and that the frequency of variant selection may reflect an interplay between two or more independent mechanisms. Examples of initial phenotypic instability are clonal liver cell lines that frequently lose the ability to synthesize and secrete albumin after a few dozen passages in culture, and skeletal muscle myoblasts that cease to differentiate after a few transfers. Examples of phenotypic change have been reported for PC-12 cells with increased number of passages (*94*). It is likely that the origin of variants in clonal cells can be explained in terms of classic mutational mechanisms or epigenetic phenomena.

Glioma cell lines. The C6 glioma cell line was derived from a chemically induced glioma in an adult rat (*95*). These cells possess many of the regulatory control mechanisms and differentiated properties of glial cells. When dibutyryl cyclic AMP is added to primary CNS glia cultures or to glial cell lines, there is an increase in morphological complexity which is reversible upon removal (*96*). Chemicals which increase intracellular cyclic AMP produce similar morphological changes (*97*). Dibutyryl cyclic AMP has been used extensively to study the effects of various factors on differentiated glia cells (*98*). C6 cells have been used in numerous studies both of toxicity and basic cellular mechanisms. This cell line has been established as a model in which several aspects of hormonal action previously observed *in vivo* or in primary brain cell cultures can be studied (*99*). C6

glioma cells have been used to study the regulation and modulation of myelin specific genes (*100–102*). Based upon the *in vivo* experimental studies and human case reports suggesting that major target sites of lead acetate are myelinating cells of the nervous system, these cells have been used to examine the toxicity of lead acetate. Results suggest that lead has a selective inhibitory effect on an oligodendroglial function expressed in the C6 glial cell line. This was demonstrated by a dose-dependent inhibition of the glucocorticoid induction of soluble cytoplasmic GPDH at the level of transcriptional processing. Since GPDH is a specific biochemical marker for the myelin-forming cells and oligodendrocytes and is believed to be involved in myelination, the selective inhibitory effect of lead on GPDH induction is consistent with the *in vivo* observations of hypomyelination.

Neuroblastoma cell lines. Cell lines have been established from both mouse and human neuroblastomas which express several neurotransmitter-synthesizing enzymes. This feature makes them good candidates for the study of regulation of gene expression and for the establishment of cDNA libraries. The first neuroblastoma cell lines were derived over 40 years ago. They form homogenous populations, proliferate rapidly in chemically defined media, extend neuritic-like processes and contain several neurotransmitter precursor molecules such as tyrosine hydroxylase. When using neuroblastoma cells it is important, and at times difficult, to distinguish between morphologic differentiation and cytotoxicity (*103*). In pesticide research, neuroblastoma lines are used because of the presence of the target enzymes AChE and NTE (*104*). The NIE115 neuroblastoma, cloned from the murine neuroblastoma C1300, has been used to study features associated with organophosphate-induced delayed neuropathy (OPIDN) with an attempt to rank NTE inhibition levels after exposure to various organophosphates (*105–107*). For these reasons, it has been unofficially proposed as a substitute for the Hen test that the U.S. EPA Pesticide Registry uses to detect organophosphorus pesticides capable of causing neuropathy (OPIDN).

Human neuroblastoma cell lines are similar to mouse or rat neuroblastoma cell lines; they are generated from human tissue and may offer species-specific responses to neurotoxic agents. One such cell line is the IMR32, which differentiates upon stimulation with several drugs and expresses several neuronal characteristics and functions such as neurotransmitter synthesis and storage, receptors, and ion channels. In such cells, the specific neuronal end points examined are associated with neurite outgrowth and expression of neurotransmitter receptors. General cytotoxicity is determined as an indicator of neurotoxic specificity. Pharmacological experiments have demonstrated that IMR32 cells express surface nicotinic and muscarinic cholinergic receptors and that classical pharmacological dynamics are present at these receptor sites. These cells have been used to examine the toxicity of various metals that target the cholinergic system *in vivo.* SY5Y is a subclone of the human neuroblastoma SK-N-SH and has neurotoxic esterase activity comparable to brain, thus allowing the study of OPIDN-inducing chemicals.

Pheochromocytoma cells. The PC-12 clonal cell line was established from a rat pheochromocytoma (adrenal medullary tumor) and has many properties in common with primary sympathetic neuron and chromaffin cell cultures (*94,108*). These cells can be induced to differentiate in the presence of NGF to resemble sympathetic neurons, to extend neurites, increase tyrosine hydroxylase (TH) activity, and dedifferentiate with the removal of such growth factors [for review see Fujita et al. (*109*)]. The PC-12 line has been used extensively to examine the mechanisms underlying NGF response and nerve differentiation. Depending on the conditions, PC-12 cells synthesize AChE, acetylcholine, and choline acetyltransferase as well as release dopamine, norepinephrine, and acetylcholine. They contain sodium, potassium, and calcium channels, and various other membrane receptors including receptors coupled to G-proteins. For these reasons they have been used extensively to examine the basic biology of neurotransmitter biosynthesis and secretion, neuronal differentiation, calcium ionic flux, and signal transduction mechanisms. They provide a useful model for studying processes associated with neuronal differentiation, synthesis, storage, and release of neurotransmitters, function and regulation of ion channels, and interactions of compounds with membrane-bound receptors. In toxicology, PC-12 cells have been used to evaluate chemical induced alterations in calcium channels neurotransmitter release and receptor, and by both biochemical and physiological methods (*110–113*).

ADDITIONAL CULTURE SYSTEMS. Microcarrier techniques have been developed to study different cell types *in vitro* (*114*). These microcarriers have a very large surface-to-volume ratio and can be used to obtain high yields of cells from small culture volumes. This system allows for mixtures of cell types to be produced at any defined ratio and as such offers an attractive model for examining specific questions concerning cell–cell interactions. Microcarrier techniques have been applied to study fiber outgrowth, synaptogenesis, myelin formation in neural cells, and metabolic interactions between cells.

Transgenic mice carrying target constructs of the polyoma large T or SV40 large T gene, have been used to establish both astroglial and neuronal cell lines. Recent developments in cell fusion techniques have allowed neuronal cells of defined origin to be "immortalized" by fusion to a transformed cell line. For example, the NG108-15 hybridoma was developed by fusion of the mouse neuroblastoma clone N18TG2 with the rat glioma clone C6BU1 using Sendai virus (*115*). This hybridoma offers the characteristics of nerve fiber extension, synapse formation, choline acetyltransferase activity, and receptors for various neurotransmitters (*116*). The NSC-34 spinal cord neuron × neuroblastoma hybrid cell line has been used to evaluate chemicals that have an *in vivo* site of action at the motor neuron (*117*). These cells have been produced by fusion of motor neuron-enriched, embryonic mouse spinal cord cells with mouse N18TG2 neuroblastoma cells (*117*). The cultures contain two populations of cells: *a*) small, undifferentiated cells that have the capacity to undergo cell division; and *b*) larger, multinucleate cells that express many properties of motor neurons. These cells proliferate in culture, but also express a number of motor neuronlike properties without the need to add inducing agents like NGF to the culture medium. Such processes include extension of neurites; generation of action potentials; expression of neurofilament triplet proteins, neuron-specific enolase and choline acetyltransferase; synthesis and storage of acetylcholine, induction of acetylcholine receptor aggregates on co-cultured myotubes; and expression of a receptor for the neuromuscular junction-specific basal lamina.

The NSC-34 cell responds to agents that affect voltage-gated ion channels, cytoskeletal organization, and some components of axonal transport. Investigation into the use of these cells as a test system for neurotoxicity examined chemicals that exert their neurotoxicity by altering specific neuronal functions: action potential

production, axonal transport, neurofilament organization, and neurotransmission (*117*). The cytoskeletal proteins in NSC-34 cells are sensitive to 2,5-hexanedione in the formation of intermediate filament aggregates. The timing for such studies is critical—as the expression of vimentin decreases with differentiation, the sensitivity of these filaments to aggregation by 2,5-hexanedione decreases (*118*). Chemical-induced changes linked to axonal transport have been examined only with sodium pyridinethione. In this case, retraction and focal swelling of processes was observed at only the highest dose and may be a manifestation of cytotoxicity rather than an alteration in organelle transport. The cells detected chemical-induced changes in voltage-gated ion channels although there were quantitative differences from primary motor neurons. These cells do not form functional synapses with cultured myotubes and therefore are not a test system for evaluating synaptic transmission (*117*). Most interesting is the fact that the NSC-34 cell does not require chemical induction to express a differentiated phenotype, providing an advantage for cell biological evaluations. However, the inability to prevent differentiation is also a disadvantage in that the population of cells most likely to differentiate is gradually diminished with successive subculture. Although fusion techniques can create such hybrid cells, many of the neuronal characteristics are lost from such preparations after a certain number of passages. Attempts have been made to transfect primary cells with oncogenes but these cells usually only survive for a short time.

End Points of Neurotoxicity

The approach used most often to discriminate between neurotoxicity and general toxicity is to examine specific end points unique to nervous system function and compare the exposure relationship between these end points and other nonspecific end points that would be ubiquitous to all eukaryotic cells. Another approach has been to compare toxic responses between neuronal and nonneuronal cells. A number of end points have been proposed as simple and rapid methods to assess chemical toxicity *in vitro*. Many end points proposed to detect neurotoxicity make the primary assumption that neurotoxicants affect specific nervous system functions. The following sections will review various measurements of basal cell functioning and end points that may be unique to the nervous system and target sites of chemical perturbation.

Basal Level of Cell Functioning. Basal level end points reflect generic cell functioning necessary for cell survival. Although basal cell function assays are used as an index of cell lethality, these measurements do not necessarily give an indication of the type of cell death. Measurements of cell toxicity include evaluation of macromolecular synthesis, lipoperoxidation and generation of reactive oxygen species, indicators of mitochondrial and lysosomal activity, loss of ions or cofactors, induction of apoptosis and DNA integrity, energy regulation (oxidative–reduction status), generic cell functions (respiration, ion transport, and protein and DNA turnover), cell–cell communication via gap junction integrity, biosynthetic reactions, transport processes, and specific enzyme changes. Although these processes can be used to assess the status of the cell, basal cell function is often limited to assays such as vital dye uptake, mitochondrial viability assays, total cellular protein, or lactate dehydrogenase release. Vital dye exclusion assays are based on the ability of cells with damaged leaky membranes to allow the entry of stains such as Trypan Blue, or the loss of intracellular stains such as neutral red. Although the vital dye assays are used as an index of cell lethality, what they actually measure is cell membrane permeability. Since cells can survive some degree of membrane permeability, these measurements do not necessarily equate with cell death. In addition, methods such as Trypan blue exclusion require a quantitation of cell numbers containing the dye. The neutral red uptake and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assays have been widely used as semispecific indices of aspects of cellular function (*119*). These indices can provide measures of cytotoxic potential that are more sensitive than vital dye exclusion. Any microscopic quantitation can prove to be problematic with cells forming clusters or cells that have a flat morphology such as astrocytes or fibroblasts. With fluorescent dyes such as ethidium homodimer and propidium iodide also excluded from live cells, a fluorescence-activated cell sorter can count the number of fluorescent cells automatically. For this, however, the cells have to be detached from the substratum and/or have to be dissociated into single cells. Another method is to measure total culture fluorescence before and after destruction of the cell membrane. As such assays require washing to remove excess dye, cells that lose adherence or disintegrate would be removed from the final determination.

The cytosolic enzyme LDH has been proposed to monitor toxicant exposures that last for days or weeks. It is assumed that over time the enzyme marker accumulates in the medium and that a stable reading of this accumulation should be available at the end of the exposure. It must be noted that LDH is not a stable enzyme and its half-life can be influenced by other products released by cells. In all cases, any measurement of a substance released by the cells into the medium must be examined relative to the total amount available for release. Therefore, such studies would determine the amount of the marker both in the medium and inside of the cell and express the data as a ratio of released/total marker. One must consider other factors that may come into play, especially in a mixed cellular culture, such as a concomitant induction of endogenous substances. Such substances could degrade proteins in the medium and influence any protein measurement of leaky membranes. Healthy cells uptake such substances as radiolabelled proline, adenosine, and chromium; elevated release can be a reliable and easily monitored index of toxicity. For measurements of ^{51}Cr release, cells are preloaded with ^{51}Cr and the amount released into the medium after toxicant exposure is measured in a gamma counter. The relative amount released as a fraction of the total amount in the preparation is the measure of cytotoxicity. All of these measures reflect the permeability of the membrane and do not directly confirm cell death.

Cytotoxicity and viability end points provide information on the intrinsic toxicity of chemicals. They have limited ability, if any, to predict neural-specific effects. However, such end points must be included to determine the health status of the cells at the time of process evaluation and possibly to differentiate specific effects from general cytotoxicity. In many cases a distinction is made based on the selectivity of the endpoint examined. For example, differentiated cell functions such as axonal transport, synaptogenesis, myelination, enzyme activities, and neurotransmitter function could be examined for neurotoxicity. Characteristics of neurotoxicants could include neurotransmitter-specific accumulation, release, or metabolism by the neural cells. The development of early and cell-specific indicators of the reaction of neuronal cells and astroglial cells to injury will require examination of neuronal and glial-specific protein changes. It is commonly acknowledged that given the complexity of the nervous system, no single

endpoint can encompass the range of neurotoxic targets. A suggestions to overcome this complexity has been to include a variety of multiple end points. Again, given the complexity of the nervous system, the ability to select such multiple end points to reflect the dynamics of the nervous system is limited by our current minimal understanding of mechanisms associated with neurotoxicity *in vivo*.

Target Sites of Neurotoxicity. In the attempt to develop *in vitro* systems to evaluate the neurotoxic potential of chemicals, one should identify *in vivo* target sites or processes, understanding the biological details associated with the *in vivo* process and the types of perturbations that can be induced on any one particular target by various chemicals. A model system cannot be developed in isolation. Without an understanding of the basic biology, the points at which the basic process can be altered, and the types of injury, a model system with any usefulness or meaning cannot be developed. In fact, the development and use of an inappropriate model, whether *in vivo* or *in vitro*, can cause damage by providing potentially biologically invalid experimental data. A number of biological processes that are target sites for neurotoxicants *in vivo* are easily examined with *in vitro* systems. The following examples will attempt to address some of these proposed processes.

STRUCTURAL. Morphological end points including effects on the cell body, axons and dendrites, can be crucial in the assessment of chemical-induced alterations in target cell populations as different morphologic patterns of chemically induced damage can be observed *in vitro* (*120*). Morphologic lesions, however, have received only limited attention as *in vitro* end points of neurotoxicity. The interpretation of altered morphology in neural cell cultures is hampered by the paucity of descriptions regarding normal cytology, the range of possible cellular lesions, and the types of possible artifacts which may occur.

Neurons exposed to substances in culture often develop structurally abnormal neurites. A common phenomenon is beading, which is the formation of gross organelle-filled dilations spaced at intervals along the neurite. Beading occurs in cells exposed to colchicine (*121*), acrylamide (*122*), or ethylene oxide (*123*). Morphological alterations can be seen in astrocyte cultures in response to chemical agents. For example, trimethyltin alters astrocyte morphology, as demonstrated by a retraction of processes and a central focus and increased size of the cell body (*37*). Microglia may be induced to differentiate into an ameboid form and initiate phagocytosis (*124,37*). All neural cells may be induced to change morphological phenotype in the retraction of cellular processes along the substratum.

Neurite Outgrowth. Neurite extension is a fundamental property of neuronal cells *in vitro* and depends upon a number of critical cellular processes such as axonal transport. Although time-consuming, quantitation of neurite outgrowth is a relatively easy task. In addition, neurite outgrowth can be determined by the measurement of neurite-specific structural elements, for example, microtubule-associated proteins, neurofilament proteins. The ability of biological factors, pharmaceutical agents, or environmental and industrial compounds to interfere with neurite outgrowth is often used to screen activity of new substances. In many cases, one examines a substance's ability to stop ongoing normal outgrowth or the induction of cells to retract neuritic processes. Assays for neurite-promoting factor, neuronotrophic factors, and glial maturation factors are often used to assess effects of exogenous substances on developmental processes. It has been proposed that one may examine a compound's ability to block biologically induced neurite outgrowth as can be seen in nerve growth factor-treated cells (*125,126*). However, this approach is limited because the inhibition of induced outgrowth may be due to indirect effects resulting from the absence or modification of certain enzymes or structural elements rather than from an effect on the cells' ability to generate neuritic processes. Although this approach may offer information of scientific interest, it may not address the issue of a direct effect of a chemical on neurite outgrowth.

Myelin. One major interaction between neurons and glia that results in a structural component of the nervous system is the process of myelination. There are a number of disease processes and compounds that will selectively alter myelin whether as a hypomyelination, a dysmyelination, or a demyelination [for review see Quarles et al. (*127*) and Morell (*128*)]. Myelination is specifically altered by triethyltin and hexachlorophene, by developmental exposure to inorganic lead, and by undernutrition. *In vivo* research efforts in this area range from morphological examination of myelinated tracts and biochemical evaluation of myelin proteins, to examination of alterations in the regulation of myelin-specific genes.

PERIPHERAL NERVE MYELIN. Because of availability of peripheral nerve tissue, much of the work on processes of myelination both *in vivo* and *in vitro* has focused on the myelination of the sciatic nerve by Schwann cells. The difficulties in culturing Schwann cells are similar to other primary neural cultures except for those associated with myelination in culture. Stimulating the cell to myelinate an axon is a very complicated task that has been accomplished by only a few laboratories engaged in mechanism-based research. Even in these cases, the number of myelin lamellae (wrappings) is limited—a significant difference from the *in vivo* condition. *In vivo* once Schwann cells are in their final location on the axon, they stop dividing and begin to elaborate myelin around the nerve (*129*). The presence of myelin along the axon serves to increase the velocity of signal conduction along the nerve and reduces the energy requirement for conducting the impulse. *In vivo* Schwann cells make galactocerebroside and the myelin-specific protein P_0 after they have migrated down the nerves, ceased dividing, and are developmentally committed to myelination (*130,131*). When they are cultured in the absence of nerve, they initially contain galactocerebroside and P_0, but the number of cells containing these molecules declines with a half-life between 2 to 3 days. Given an increase in cell number, this decrease is not due to cell loss but rather a progressive loss of antigens. Much research effort has been directed toward developing a model system for myelination to understand its basic underlying requirements. After 15 to 20 years work, we now know more about the interactions between the myelinating cell of the peripheral nervous system and its environment. However, the culture system is a very demanding system that has to date only been applicable to mechanistic-based research and is beyond the demands of a screening test for neurotoxicity.

CENTRAL NERVOUS SYSTEM MYELIN. Efforts to develop a system to examine CNS myelination by oligodendrocytes have a much shorter and complicated history. For example, unlike the PNS where the relationship between Schwann cell and axon is 1 to 1, the relationship between oligodendrocytes is 1 to 40 separate nerve fibers. In addition, not all Schwann cells and oligodendrocytes produce a myelin sheath. One model recently adapted to examine CNS myelination is the cerebellar

slice culture with a modification of substratum attachment rather than a roller bottle method (*132*). It is still too early to determine whether this model will offer a method to evaluate toxicant-induced alterations in myelin. A model system to evaluate both the process of myelination and structural myelin wrappings would be beneficial in understanding the action of neurotoxicants that selectively target myelin. However, critical *in vivo* target sites for neurotoxicants are absent, i.e., multiple lamella and nodes of Ranvier (spaces along the axon between each cell's myelin wrapping). For example, triethyltin and hexachlorophene both produce alterations in myelin as evidenced by a splitting of the intraperiod line (the space between myelin lamellae) (*133*). Without multiple wrappings a similar effect could not be seen *in vitro*. Chemicals that produce hypomyelination have a better chance of being detected. However, in many of these cases, such as in the developing nervous system after lead exposure, the myelin that is produced is normal in protein and lipid content; there are just fewer lamellae and thus less myelin (*134,135*). *In vivo* efforts suggest that the perturbation is on the myelin sheath and not a direct effect upon the myelinating cell. This also would limit the usefulness of a culture system that is restricted to the cell body and to very little if any plasmalemma in the form of myelin.

Although chemicals and disease processes have been classified as primary demyelinating agents, this classification system is based upon a morphological criteria (*136*). It does not take into account the role of dynamic signaling interactions between the myelin sheath and its underlying axon. Whether the resulting pathology in the myelin sheath is due to a direct effect on myelin or the myelinating cell or in response to a perturbation in the underlying axon is still a major question in whole animal models [for review of neuropathies see Pleasure (*137*)]. Additional yet critical players to the morphological end product seen *in vivo* are both the resident and the infiltrating macrophages, which have the role of stripping myelin from the axon and removing it from the nerve area. Any *in vitro* model system designed to determine effect of a toxicant on myelin would need to include all of the known *in vivo* active participants.

Existing *in vitro* systems allow examination of the signaling mechanisms linked to initiation of myelination in the developing organism. However, given the difficulty of the culture, such efforts should be limited to well-defined, focused questions aimed toward understanding the mechanisms associated with perturbations to developing myelinating cells. Efforts have been made to develop an *in vitro* system to model the vulnerability of the myelin genes in the developing brain. Using mixed glial cultures to preserve the temporal specificity of the myelin gene expression, Royland and co-workers (*138*) developed a system to examine the effects of nutritional deprivation on myelin gene expression. When the mixed glial cultures were maintained in defined medium, myelin genes responded to various manipulations such as alterations in glucose concentrations. The cultures can be further manipulated to produce cultures of purified oligodendroglia, allowing for more detailed examination of the mechanisms associated with alterations in the expression of specific myelin genes during development. These cell culture systems preserve the *in vivo* mechanisms of activation and upregulation of myelin genes critical for myelin development *in vivo*. Thus the primary cultures allow for further examination of toxicant-induced alterations in myelination at the *in vitro* level and can provide an additional experimental system for studies of normal and abnormal gene expression during development of the brain. Aggregating cell cultures also provide a useful system for biochemical and morphological analysis of myelin and the process of myelination and remyelination (*75,139–141*). The morphological characteristics in reaggregates are similar to those *in vivo*, and myelin protein can be isolated in sufficient quantities for biochemical analysis. In addition, the yield of the myelin-associated enzyme CNP and the myelin protein composition is similar between 30-day aggregate cultures and adult rat brain (*75*).

FUNCTIONAL END POINTS OF NEUROTOXICITY. *Axonal transport.* Axonal transport involves mechanisms similar to those that all eukaryotic cells use to transport materials from their sites of synthesis to their sites of utilization and subsequent sites of degradation. Alterations in this process have broad implications for the biological functioning of the organism (*142*). A key question surrounding toxicant-induced alterations in axonal transport deals with whether the effect occurs by direct or indirect mechanisms. A toxicant can produce an effect on transport via several mechanisms. It might interact with a specific step in the mechanism of axonal transport, directly affect the movement of a specific transport component without altering transport per se, or alter the spatial arrangement or production of axonal components. Additionally, an effect might be due to a more general biochemical disruption such as energy metabolism. The majority of approaches used to examine toxicant-induced alterations in axonal transport have involved whole animals. Use of *in vitro* techniques are now possible with the establishment of the video-enhanced contrast differential interference contrast microscopy (VEC-DIC) methodology to examine organelle movement along the axon. The N1E115 murine neuroblastoma cell line has been used to study perturbations of differentiation and axonal transport (*143–145*). When the cells are exposed to serum-free medium, they extend large, stable neurites that are well suited to analysis of intraaxonal movement of organelles by VEC-DIC. Axonal transport is determined by the number of organelles traversing a line perpendicular to the long axis of the neurite; velocity is determined by following individual organelles moving along the neurite. This method has been successful in detecting a significant reduction in bidirectional organelle flux induced by vinblastine, an antimicrotubule drug that impairs fast axonal transport *in vivo* (*143*). Examination of acrylamide, a neurotoxicant that affects aspects of axonal transport, showed a toxicity to differentiating neuroblastoma, but organelle movement remained unimpaired. Comparison with *in vivo* results raises the possibility that rapid transport of radioactive markers could be altered without impairment of vesicular traffic and lends support to the observation that a subset of axons or organelles is impaired by acrylamide (*146*). These types of defects would not be readily apparent using video microscopy if the remaining organelles moved normally. Therefore, such an *in vitro* system would be limited to detecting perturbations that affected all populations of axons or neurites. This would be inconsistent with the *in vivo* observations of differential vulnerability of sensory or motor nerves to various neurotoxicants. When these cells were examined for their ability to detect the toxicity of acrylamide, no alterations in cell morphology were seen until 2 weeks after exposure to 25 μg/ml acrylamide. In the exposed state, the growth cone on the leading edge of the outgrowth process was defective and neurite swellings were noted (*147*). Similar alterations in growth cone integrity have been reported in N1E115

cultures, chick clonal root ganglia cultures, and chick spinal ganglia. Walum et al. (*122*) showed that acrylamide exposure of NIE115 cells produced neurite swellings initially in the distal regions followed by progression to the proximal portion. This is similar to the distal dying-back axonopathy observed *in vivo* following acrylamide exposure. The level of sensitivity of the cells was determined with comparison of eight cell lines (neuroblastoma 41A3, neuroblastoma N18, neuroblastoma N1E115, neuroblastoma × glioma hybrid NG108CC15, glioma 13BMG, glioma C6, fibroblast RLF, and fibroblast RMC). Of all these cells, the N1E115 and NG108CC15 cells, which are considered the most differentiated of the neuronal cells tested, were found to be most sensitive based on growth characteristics and morphology.

Electrophysiological indices. Receptor and ion channel expression can be studied *in vitro* using electrophysiological recording, although specific subtypes are best characterized by single-channel recording. Such detailed recording requires cell membranes free from debris, which may be technically difficult to achieve with organotypic or explant cultures. In addition, synaptic function and spontaneous activity in neuronal networks can be readily monitored in any system sufficiently complex to show such activity. The net result of complex functional relationships may be evaluated by relatively simple electrophysiological techniques such as paired pulse inhibition and long-term potentiation.

Blood–Brain Barrier. The blood–brain barrier (BBB) both protects and nurtures the brain (*26,148*). The endothelial cells in brain microvessels are sealed together by tight junctions and this cuff restricts the passage of most polar and organic molecules from the bloodstream to interstitial fluid of the brain (*149*). Molecules pass through the BBB by the aid of protein and amino acid transport enzymes and carriers such as γ-glutamyl transpeptidase (GGTP), alkaline phosphates, and glucose transported enzyme (GLUT-1) (*150–152*). These endothelial cells of the BBB are physically in contact with astrocytes that influence many of their functions (*153–155*). The astrocytes regulate the rigidity of the tight junctions, thus producing a higher electrical resistance across the cells. They can enhance a variety of enzyme activities such as GGTP, an amino acid carrier, and regulate the expression of GLUT-1 protein. Thus, the BBB can be disrupted either by damaging the endothelial cell or by interfering in the interdependency of astrocyte and endothelial cells.

Although it has been clearly demonstrated that toxicity is dependent on the sensitivity and developmental stage of target cells, concentration of test compound, and duration of exposure, it has been proposed that any chemical that passes the BBB is neurotoxic (*156*). Since many chemicals gain access to the brain parenchyma by disrupting the BBB *in vivo* (*157–162*) attempts have been made within *in vitro* neurobiology to develop a model test system. Questions concerning the BBB have focused primarily on the use of isolated microvessels prepared from bovine and rodent tissues (*26,153*). One recently developed system (*163*) is a co-culture of a bovine aortic endothelial cell line (or a rat brain endothelial cells) with rat brain astrocytes (or C6 rat glioma cells) in a tridimensional hollow-fiber culturing apparatus. The system is maintained under pulsatile flow conditions to mimic intraluminal blood flow and to allow for chemical delivery. One cell line that has been proposed as a model test system for the BBB is the Madin-Darby canine kidney cell line. This line displays a variety of functional and enzymatic similarities to endothelial cells of the BBB including electrical resistance, tight junctions, and glucose transporter enzymes, offering a number of potential target sites for examination.

Signal Transduction Mechanisms.

CALCIUM HOMEOSTASIS. The normal functioning of the neural cell is critically dependent on the intracellular distribution of calcium with cytosolic-free calcium functioning as the critical pool for regulation of intracellular events (*164,165*). The viability of the cell can be affected when the mechanisms of calcium homeostasis fail (*166–168*). If this highly synchronous and coordinated process fails, there is an increase in cytosolic-free calcium, which can activate a number of intracellular reactions. Such reactions include the release of neurotransmitter, phosphorylation of proteins, and protease activity (*167,169–171*). Intracellular calcium also plays a role in the control of neuronal growth and associated growth cone activity. Many neurotoxic agents with diverse mechanisms have been reported to perturb intracellular calcium. Included in these active chemicals are methyl mercury, chlordecone, triethyl lead (*172–174*), cyanide (*175*), pyrethroids (*176,177*), methyl mercury (*168,175*), PCB congeners (*178,179*), and aluminum (*180–182*).

NEUROTRANSMITTERS AND HORMONES. Numerous culture systems have been developed to address specific biological questions with regard to nervous system cellular induction of or responsiveness to neurotransmitters or hormones. With regard to neurotransmitter synthesis, clones fall into four subsets: cholinergic; adrenergic, both cholinergic and adrenergic, and neither neurotransmitter. All four subsets synthesize and secrete large amounts of AChE into the culture medium. Synthesis of both catecholamines and acetylcholine (ACh) tends to increase with morphological differentiation of the cells, although there is no evidence of an exogenous induction of morphological differentiation producing either an increase or decrease in neurotransmitter synthesis. The first clonal cell lines established in culture were derived from C1300 mouse neuroblastoma. Given that this tumor had been passaged for 30 years prior to growing in culture, and that cell lines derived from mouse tumors generally have very unstable karyotypes, the hundreds of clones isolated from the original tumor have widely different characteristics. Neurotransmitter synthesis in C1300 clones is limited largely to ACh and/or catecholamines due to the origin from cells of the neural crest. Cell lines derived from human neuroblastomas also synthesize predominantly ACh and/or catecholamines (*183*).

Although the numerous mouse-derived neuroblastoma cell lines available could offer a culture system to examine alterations in ACh or catecholamines, they offer very little in other neurotransmitter systems. Rat clonal cell lines offer increased diversity of neurotransmitter systems. These cells make primarily the inhibitory neurotransmitter GABA acid, with a limited number of cells making small amounts of ACh and catecholamines (*184*). Additional work by Kimes et al. (*185*) demonstrated that subclones were able to synthesize two or more neurotransmitters, confirming that individual CNS cells, like those from the neural crest, are able to make multiple neurotransmitters. The clonal PC-12 cell line also has the ability to synthesize both ACh and norepinephrine (*186*). Data on polychlorinated biphenyls suggest an effect on catecholamine metabolism in PC-12 cells similar to CNS dopamine metabolism. This model has been continued to examine the structure-activity relationships among PCBs and catecholamine metabolism. Another group of cultured cells able to synthesize multiple neurotransmitters is the sympathetic

ganglia. Dissociated sympathetic cervical ganglia cells from newborn rats are able to synthesize both ACh and catecholamines (*187–190*).

Cell cultures represent an excellent system in which to study trophic influences of growth factors and other compounds, as the cholinergic neurons are easily assessable for manipulation and observation. For example, cultures of dissociated cells from the septal area of fetal rat brains have been used to study the effects of NGF, thyroid hormones, and gangliosides on septal cholinergic neurons (*191*). Isolated adrenal medullary chromaffin cells cultured in defined medium are useful for studying the regulation of tyrosine hydroxylase, dopamine β-hydroxylase and phenylethanolamine-*N*-methyltransferase, the key enzymes in catecholamine biosynthesis [for reviews see Furshpan et al. (*189*) and Bunge et al. (*192*)]. This culture preparation usually require a retrograde perfusion of the intact bovine adrenal gland with collagenase, followed by separation of medulla from cortex, and enzymatic and mechanical dissociation of medullary tissue (*193*). The ability to induce ACh synthesis is significantly decreased with increased age of the animal from which cells are obtained (*188*).

The avian ciliary ganglion represents the most studied model of a selected population of cholinergic cells in the peripheral nervous system (*194*). The development of cholinergic enzymes, the effect of denervation, and the effects of aging on gangliahave been reported (*195,196*). The avian system has several advantages. Its developmental and aging process is well characterized; its size and location make it easy to dissect; it is comprised of a relatively large and homogeneous cell population and it can be studied both *in vivo* and *in vitro*. In the chick, the ciliary ganglion is located 3 to 4 mm from the eye, deep in the orbit, on the branch of the occulomotor nerve that innervates the oblique muscles of the eyeball. In addition to the branch of the occulomotor nerve, ciliary nerves and choroid nerves emerge from the ganglion, providing innervation to the oblique muscles of the eyeball, smooth muscles, and striated muscles of the iris.

Based on immunocytochemical detection of TH and serotonin (5-HT), techniques have been developed for dissection of monoamine nuclei from embryonic brain tissue for dissociated cell cultures, explant cultures, or transplant cultures. Although regions containing TH or 5-HT immunoreactive cells can be delineated as early as embryonic day 12 (E12), it is very difficult to free the neural tube from the surrounding tissue without damage. The earliest stage allowing for dissection is E13. With increasing age, the dissection becomes easier. However, the yield of viable cells decreases rapidly and only one ganglion can be obtained per animal. Thus, the optimal time for tissue collection is E13 or E14 (*197*). Whitaker-Azmitia and Azmitia (*198*) reported effects of various serotonergic drugs, drugs of abuse, and other therapeutic agents including antidepressants on a preparation of dissociated primary cells from midbrain regions rich in serotonin neurons. One striking aspect of the data was the variability in the results for any given substance such that the results of uptake of radiolabeled serotonin were presented as qualitative changes rather than a quantitative measurement. Factors contributing to this variability were time in culture, plating density, and presence of steroids or glucose in the media. Based on the effects of amphetamine on brain catecholamine function, NG108-15 neuroblastoma–glioma cells were used to study the involvement of the serotonergic neurotransmitter system in toxicity induced by amphetamine and its various derivatives. [^{3}H]*d*-Amphetamine uptake and cell viability demonstrated a parallel dose-related decrease after exposure to amphetamine and its various derivatives (*199*). Cell death as determined by these two measurements could not be blocked by the specific serotonin uptake inhibitor paroxetine, which at higher doses was also toxic to the cells. The ability of the cells to uptake amphetamine and to show a differential pattern of viability response to various derivatives supported the use of this culture system to screen for the neurotoxic effects of drugs of abuse. However, caution is necessary prior to any widespread use of this approach, as the system was unable to detect any differences between the *d*- and *l*-isoforms of amphetamine which *in vivo* have distinctly different effects on the nervous system: the *l*-isoform has psychoactive properties and the *d*-isoform lacks such activity.

The sexually dimorphic nucleus of the preoptic area is an example of a morphological gender difference in the rat hypothalamus and has been a valuable model for the study of neural, cellular, and molecular mechanisms by which gonadal steroids influence the nervous system. The nucleus is composed of a heterogeneous population of neurons situated in the medial preoptic area of the brain. The nucleus receives and projects to numerous regions within the limbic system, including connections to most hypothalamic nuclei, and various nuclei within the brain stem (*200*). This system offers a wealth of characterization on the effects of steroids, the developmental critical period for steroid action and identified markers for neurons of the nucleus. It has been used as a model for the study of hormonal regulation of gene action, which can influence survival, differentiation, and expression of neuropeptides and receptors. This system may offer a model to examine the effects of various estrogenic-like compounds currently of concern for developmental neurotoxicity. Unfortunately, only one tissue punch per region can be obtained for culture from either adult or developing animals.

For the study of fully differentiated neurons, methods have been developed to isolate neurons from the mature mammalian brain. Usually, such methods are used to isolate neurons for electrophysiological measurements and not for culture. It is possible to isolate neurons from small circumscribed regions of the brain which preserve dendritic structure, thus allowing identification of morphological class. Neurons can be isolated with intact synaptic boutons exhibiting spontaneous release of excitatory and inhibitory neurotransmitters (*201*). The procedure yields approximately 10 to 20% of the original cells and would allow for investigation of specific neurotransmitter-containing neurons.

Reactive Changes. Reactive changes can be useful end points of toxicity as they can be used to determine threshold concentrations of toxicity or to identify sensitive systems prior to more mechanistic analysis. GFAP is widely used as a marker of toxicant-induced astroglial reaction *in vivo* and has also shown value *in vitro* (*202*). GFAP levels can be increased in the early stages of a response, whereas a decrease is seen with increasing cytotoxicity (*203*). Such biphasic reactions are common and can lead to problems in interpretation of results. Inhibition of the reduction of MTT has been widely used as a measure of impaired mitochondrial or cytoplasmic reduction capacity, but many agents produce a reactive stimulation of MTT reduction at low doses, followed by a decrease at higher, cytotoxic doses (*204*). Biphasic responses are also seen in a time-dependent manner. The astrocytic toxin dinitrobenzene decreases the glutathione content of primary astrocytic cultures within 2 hr while at subcytotoxic concentrations a larger reactive increase is seen by

24 hr (*205*). Therefore, both time and concentration dependency must be established before data interpretation is possible.

Even within common neurotoxic end points, there is considerable variation between chemicals that target a specific process. Current information on sites of action of known neurotoxicants can be helpful in determining the types of target sites to examine. However, such information can be misleading in that it offers the impression that one would be able to determine the potential for neurotoxicity in new and untested chemicals using only current information. Although this may be the case, the techniques to evaluate these target sites are sophisticated and require not only technical training but also a biological understanding of the system in order to conduct meaningful scientific investigation, even at a preliminary level. In neurotoxicology, difficulty arises when attempting to determine which target sites should be used to evaluate neurotoxicity of a chemical with unknown biological properties. Attempts to distinguish between chemicals that produce cytotoxicity versus a selective neurotoxicity in any culture system is hindered by the identification of a suitable global end point for neurotoxicity that can be examined within the culture system of interest. With current advances in cell biology and neurosciences, additional elements of cell processing are being identified which may allow for a limited number of common mechanisms of cellular dysfunction to be identified. Until that time, efforts to understand how neurotoxicants exert an adverse influence on the various cells of the nervous system will continue, based upon current knowledge of neurobiological functioning, *in vivo* analyses, previously identified sites of action for numerous chemical substances, and a great deal of hard work.

Quantitative Analysis

The usefulness of an experimental model, whether *in vivo* or *in vitro,* can be assessed from many factors such as reproducibility, sensitivity and specificity, or other measures which indicate how well the simplified system describes the biological process in qualitative and quantitative terms. The result of such assessment is predictability. What is frequently implied in toxicology is that the ultimate objective of the model experiment is to predict an effect in man. Validation should be done by analyzing the biomechanisms at work in the model system and comparing them with the much more complex inner workings of the whole organism. Usually the scope of such a comparison is limited. Therefore, we are forced to limit the validation process to a comparison of the outcome of the model experiments with the results of a comparable challenge of the particular biological system. Within the context of test development and acceptance, the word "validation" has two definitions. One refers to the validation of the test in that it addresses such issues as robustness and reproducibility within laboratories, repeatability between laboratories, standardization of protocols, and decision-making criteria. All of these factors are independent of the biological relevance of the test.

The other definition refers to the validation of the end point. In this context, one determines whether the test can measure or predict what it is designed to measure, and whether the end point is mechanistically related to the effect of interest, including criteria for interpretation of results. Some models may be biologically useful but not predictive. In this process one would need to use analogs that are active and inactive with respect to process, and examine effects in context of general toxicity.

Because multiple samples can be generated from any one specific source, whether a primary culture, cell line, or brain slice, it is critical that each distinct preparation be considered as the statistical unit (i.e., *n* for statistical analysis). For example, even though a number of brain slices can be obtained from any given animal, the source remains one animal and the animal remains the statististical unit. The same is true for a primary culture requiring a pooled number of animals. The individually plated wells all represent one preparation, an *n* of 1. Within such preparations, it is usually considered good experimental protocol to assay each component of the evaluation in triplicate. Such procedures are standard in most biochemical assays.

The description of a dose–response curve obtained from *in vitro* experiments is generally based on a linear regression analysis, plotting the logarithm of the dose on the horizontal axis and the response on the vertical axis. Although this method allows for calculation of an effective dose, it does not allow for examination of the slope of the dose–response curve. Each of these values is important and can be manipulated by the choice of data points. Such linear regression analysis can not describe complex dose-response curves such as those showing a biphasic response. For a review of mathematical approaches to determine dose–response relationships *in vitro*, see Bruinink (*32*).

Strategies in Applying *in Vitro* Techniques in Neurotoxicology

Goldberg and others have suggested that development of *in vitro* test models should progress from the empirical to the more defined (*206–209*). The questions are initially unfocused and the methodologies heuristic. The subsequent *in vitro* models are designed to specific *in vivo* targets and incorporate mechanistically based end points. Scala (*210*) proposed a three-phase validation of *in vitro* models, with each stage addressing a different degree of interpretability and reliability. In this approach, *in vitro* models are initially established with mechanistic end points incorporated. A limited number of chemicals known to target that mechanism are first tested. Chemical selection for this initial stage is used to define the relevant end points and as a quality control standard. For example, cell lines or primary cells that contain a target enzyme (i.e., AChE, NTE) for many pesticides could be used to develop an *in vitro* system to assess a specific process identified *in vivo* as a neurotoxic mechanism associated with these pesticides.

Veronesi (*211*) identified the need for a variety of cell culture models and multidisciplinary end points to parallel neurotoxic targets *in vivo.* This can be difficult because few specific targets of neurotoxic action are known. To differentiate neurotoxicants from other chemicals, it was recommended that nonneural cells be included in any battery to index basal-level functioning. To accomplish this task a tiered approach was proposed. The first tier would include test systems to differentiate neurotoxicants from cytotoxicants. However, even this stage would require using combinations of nonspecific and specific neural end points and batteries of neural and nonneural cell lines. The second tier would differentiate classes of neurotoxicants (metals, cholinesterase inhibitors, solvents, etc.) using specific end points that are specifically targeted *in vivo.* This could be difficult, given the variability of target site relative to age and length of exposure. For example, at high doses, carbon disulfide is acutely toxic to the CNS whereas lower doses and prolonged exposures produce a peripheral neuropathy. The third tier would address distinct questions confined to specific classes of neurotoxicants e.g., structure–activity relationships.

Preliminary evaluations of a similar screening protocol have been published (*212*). Using selected chemicals and a

range of systems and simple end points, a reasonable correlation between *in vitro* and *in vivo* toxicity was seen. However, 2 of 21 chemicals gave a false negative, and 4 positives gave an incorrect target cell type (astrocytes, not neurons). This result illustrates both the potential uses and actual limitations of *in vitro* systems as open-ended screening systems.

Many of the *in vitro* experiments that assess the toxicity of various chemicals and drugs demonstrate the difficulty of correlating *in vivo* and *in vitro* effects of neurotoxicants and the limitations of an *in vitro* model as an alternative approach for investigating complex effects at the level of the nervous system. However, they present an opportunity to use *in vitro* methodologies for studying the underlying mechanisms of action of toxicants. The information obtained from such studies can be used in the refinement of future studies *in vivo*. The use of cells *in vitro* has resulted in new insights into mechanisms potentially important in our eventual understanding of the mechanisms of action of neurotoxicants during both development and aging of the nervous system and degenerative brain disorders. Validation should include analysis of the biomechanisms at work in the model system and comparison with the much more complex inner life of the target. Usually the scope of such a comparison is limited. Therefore, we are forced to limit the validation process to a comparison of the outcome of the model experiments with the results of a comparable challenge of the particular biological system. Maintaining the requirements for determining if a chemical is neurotoxic with *in vivo* data from, the question remains whether *in vitro* systems can be developed that will be able to make the same determination.

Conclusions

Current *in vivo* animal procedures are sometimes less than ideal predictors of human neurotoxicity. There is a consensus that *in vitro* methods can provide useful information concerning basic biological processes underlying neurotoxicity and specific information concerning a chemical's mechanism of action. At this stage of methods development and validation, *in vitro* techniques can provide data that complement established *in vivo* testing approaches. *In vitro* methods are not yet capable of replacing *in vivo* tests because they do not reflect *in vivo* nervous system complexity nor assess the full range of neurobiological functioning *in vivo*, i.e., cognition, motor coordination, and sensory processing and integration. *In vitro* systems are not yet viable means of predicting neurotoxicity where the site of action is unclear or not known. Results from *in vitro* studies must be interpreted within the context of the integrated nervous system. There is greater confidence in chemically induced *in vitro* changes if they can be associated with specific neurotoxic effects *in vivo*.

It is important that procedures used in *in vitro* neurotoxicology are valid. In this context, test methods should both measure what they are designed to measure, i.e., construct validity, and have the ability to measure a characteristic relative to some generally recognized standard, i.e., criterion validity. The results of *in vitro* procedures should also be interpretable within the context of plausible biological responsiveness to toxicological exposure and possess the ability to predict neurotoxic risk in humans. The process of demonstrating predictive validity can be facilitated by selecting *in vitro* end points associated with known biological mechanisms of neurotoxic effect. Therefore, attempts to validate *in vitro* tests for neurotoxicity testing should be limited to test systems designed for and based on assessment of specific mechanisms of neurotoxicity. The sole use of open-ended general toxicity *in vitro* screens of neurotoxicity at this time does not seem warranted.

In vitro techniques have their greatest potential in experiments involving mechanistically based hypothesis testing. *In vitro* approaches could be used in several target sites where there is a significant understanding of basic biological processes including chemically induced effects on receptor-mediated changes, neurite outgrowth, electrophysiological changes, certain neurochemical end points (e.g., neuropathic esterase/acetylcholinesterase, ion channel function), and alterations in specific neurotransmitters, enzymes and/or hormones. *In vitro* procedures may be particularly useful in assessing questions concerning structure–activity relationships. Other potential target sites for *in vitro* examination include axonal transport, blood–brain barrier, growth and development, astrocytic–neuron interactions, bioactivation, free radical/antioxidant systems, xenobiotic metabolic capacity, and myelination. In each case, the choice of the culture preparations and complexity of the *in vitro* system should be appropriate for the hypothesis following additional methods development and validation tested.

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